



PHD

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**INSECTICIDE RESISTANCE IN THE
CURRANT- LETTUCE APHID,
*NASONOVIA RIBISNIGRI***

**Submitted by Matthew Barber
for the degree of PhD
of the University of Bath
2002**

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ABSTRACT

Bioassays conducted on field populations of *Nasonovia ribisnigri* (Mosley) exhibited four modes of resistance: full susceptibility; low-level pirimicarb resistance only (up to 5-fold); pyrethroid resistance only (up to 25-fold); or intermediate resistance to both pirimicarb and pyrethroids. No resistance was found to imidacloprid. Preliminary bioassays using pymetrozine showed varied performance against adult *N. ribisnigri*. Pirimicarb resistance was consistently associated with a more intensely staining esterase band disclosed by polyacrylamide gel electrophoresis (PAGE). Radiolabelling showed the elevation of esterase activity to be due to the overproduction of the esterase rather than a more active form. The esterase of interest (E0.34) was purified by PAGE and electro-elution. Incubation of E0.34 with a serial dilution of Triton X-100 showed esterase activity to increase in hydrophobic conditions. Reactivation studies showed the role of E0.34 in pirimicarb detoxification to be one of sequestration rather than rapid hydrolysis. Under overcrowded rearing conditions, E0.34 activity was reduced in pirimicarb resistant populations. Attempts to rapidly diagnose pirimicarb resistance by a total esterase assay failed to fully distinguish susceptible and resistant individuals. Involvement of a butyrylcholinesterase not associated with resistance, and possible inhibition of E0.34 by endogenous compounds released during homogenisation may have impaired the resolution of the assay. Pyrethroid resistance appeared to be associated with a different, independent mechanism, although no knockdown resistance (*kdr*) mutation was identified in the sodium channel region known to encompass *kdr* mutations in other species. Acetylcholinesterase and glutathione *S*-transferase assays failed to show any difference between resistant and susceptible populations. Field studies showed resistance to pirimicarb to be expressed as an enhanced tolerance of residues rather than through direct contact at the time of spraying. Pyrethroid resistance conferred an immediate survival advantage after direct spray contact.

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My parents for letting me get on with whatever I wanted to do.

Publications arising from this work

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- Barber, M., Kift, N., Tatchell, G.M., Moores, G., and Denholm, I.** (In Press). Outdoor lettuce: The control of aphids resistant to insecticides. Horticultural Development Council Annual Report for Project FV 210a.
- Barber, M., Moores, G., and Denholm, I.** (2002). Biochemical characterisation of pirimicarb resistance in the currant-lettuce aphid, *Nasonovia ribisnigri*. Proceedings of the IUPAC Crop Protection Conference 2002 **1**, 291.
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1. INTRODUCTION

Insect pests are a continuing problem in crop production and while biological control methods and insect resistant plant breeding provide some means of protection, chemical insecticides remain the primary defence against insect attack. The widespread use of synthetic insecticides began with the implementation of dichlorodiphenyltrichloroethane (DDT) for the suppression of mosquitoes, fleas and lice during World War II (Pedigo, 1999). Subsequently, DDT and other newly developed insecticides were implemented in agricultural pest control to help achieve the high crop yields required by the world's rapidly expanding population. However, while food demands were met in the short term, the new agricultural practices adopted during this time, and intensified thereafter, led directly to many of the ecological problems found in agriculture today, including the widespread occurrence of insecticide resistance.

There are many causes of insect control failure. For example, 'faulty equipment, insecticides of an inferior quality, or inadequate or untimely application' (Sawicki, 1987). If these reasons can be excluded then the possibility of insecticide resistance must be examined. The definition by which most researchers currently diagnose resistance is that of Sawicki (1987) who stated 'resistance marks a genetic change in response to selection by toxicants that may impair control in the field'. Reports from the field of resistance by a new insect species to pesticides are frequent and it is only by a programme of systematic bioassays and biochemical experiments that resistance can be confirmed or refuted and, in the former case, a management strategy developed.

In this project, the occurrence, mechanisms and implications of insecticide resistance in the currant-lettuce aphid, *Nasonovia ribisnigri* (Mosley), have been investigated. The following section discusses the causes, mechanisms and management of insecticide resistance in the context of other insect species.

1.1.Causes Of Insecticide Resistance

The selective pressure of insecticides causes the resistant gene(s) to increase in frequency within the population. The speed at which resistance occurs is attributable

to several factors, which can be divided into genetic, biological and operational (Georghiou, 1983).

Genetic factors are important to understand if predictions are to be made regarding the development of resistance. Forgash (1984) described the evolution of resistance in a population as a step-wise process whereby resistance genes become associated with other favourable survival genes. Resistance then gradually develops until a point is reached at which it rapidly increases. The resistance alleles can vary in dominance, frequency and number required for phenotypic expression. Brattsten *et al.* (1986) proposed that insects can use the same mechanisms that have evolved over millions of years to protect against natural plant allelochemicals and pathogens to resist synthetic insecticides. Furthermore, resistance may occur faster if an insecticide from the same group as one that has previously been resisted is used. Georghiou (1983) suggested that the often considerable increase in the occurrence of resistance in insects after the use of organic pesticides is due to the selective target site activity of the new compounds which, while safer to humans, lack the continual, multi-target-site effectiveness of the inorganic insecticides and therefore, may only require one gene alteration before resistance evolves.

Biological factors such as generation turnover, the number of offspring per generation and life cycle are all closely connected with genetic factors in determining how quickly resistant alleles become fixed in a population. Other biological factors include migration, fortuitous survival and natural enemies. Metcalf (1989) observed that natural parasites and parasitoids of insects are often destroyed by insecticides before the target insect, leading to additional, secondary pests such as spider mites and aphids becoming economically important primary pests.

Operational factors are controlled by the grower and are therefore the most convenient target for management strategies. The most common mistake made by growers is a combination of both incorrect chemical choice and inappropriate application methods. When control failure occurs, the general response is to reapply the same insecticide in higher concentrations over a shorter period (Forgash, 1984). These actions simply increase the selection pressure on the pests and speed up the rate at which resistance to one particular chemical, or class of chemical, occurs

throughout the pest population. Other factors include the relationship of insecticides to chemicals previously used on a crop, the targeting of the correct life stage of the pest and the application of the insecticide to the correct part of the plant. Additional social and economic pressures, while not directly linked to resistance, can also lead to poor agricultural practices. For example, consumer demand for pest-free crops may result in excessive spraying regimes in an attempt to remove every individual insect (Denholm *et al.*, 1998).

1.2.Mechanisms of Insecticide Resistance

Resistance can be caused by alterations in an insect's physiology, behaviour and biochemistry which can all affect the uptake, distribution, storage, metabolism and efficacy of an insecticide (Welling and Paterson, 1985). There are several methods by which these alterations can confer resistance. Behavioural mechanisms include migration to another crop or avoidance of the toxin on the same crop, for example, moving underneath a leaf. Such mechanisms are mainly associated with insecticides that have anti-feedant or irritant qualities since these do not inflict instant mortality. Further investigations on these mechanisms have been limited because of the difficulty in designing behavioural bioassays (Mallet, 1989). Similarly, the physiological mechanism, delayed cuticle penetration, has had little research conducted as it is the 'least significant as a single resistance mechanism' (Soderlund and Bloomquist, 1990) but it has been shown to exist in houseflies (*Musca domestica* L.) and certain species of Lepidoptera (Plapp, 1984). Insects can also adapt physiologically by depositing toxic chemicals in specialised tissues (sequestration) or by accelerating excretion rates (Gullen and Cranston, 1994). However, biochemical resistance is the most common type of resistance (Pedigo, 1999).

1.2.1. Biochemical mechanisms

Biochemical resistance involves preventing the insecticide from reaching its target-site, either by metabolism of the insecticide or by target-site insensitivity.

1.2.1.1. Insecticide metabolism

1.2.1.1.1. Esterases

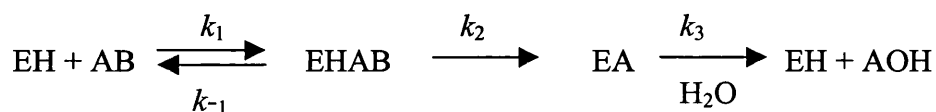
Interest in insect esterases arose with reports of resistance to organophosphate insecticides (OPs), used heavily in the 1950s and 60s. It was known that OPs caused

the inhibition of some esterases (enzymes that catalyse the hydrolysis of an ester) in insects and the direct action of these compounds was on the cholinesterase in the insects' nervous system (van Asperen, 1962). Oppenoorth and van Asperen (1960) showed that OP-resistant *M. domestica* had a low affinity for the esterase substrates methyl- and phenylbutyrate compared with susceptible strains. The mutant ali-esterase theory (Oppenoorth and van Asperen, 1960) proposed that a mutant esterase, found in resistant *M. domestica*, which exhibited a higher level of dephosphorylation than the susceptible ali-esterase, became reactivated after initial phosphorylation by an OP while its activity against the model substrate was reduced. Using a rapid colourmetric esterase assay on susceptible and resistant *M. domestica* strains, van Asperen (1962) concluded that both a cholinesterase and an ali-esterase were involved in the degradation of the substrate 1-naphthyl acetate. It was demonstrated that both enzymes had equal activity since activity was reduced by 50% when the cholinesterase inhibitor, eserine, was added to insect homogenates. The esterase assay was adapted from a method originally developed for the quantitative assay of esterase activity in mammalian tissues and blood (Gomori, 1953). The reaction involved esterases in the insect homogenate producing naphthols from the substrate 1- or 2-naphthyl acetate. These naphthols in turn react with a diazo solution, diazoblue-sodium laurylsulphate solution, to give a strong blue colour (1-naphthol) or a strong red colour (2-naphthol), the intensity of which could be read using a spectrophotometer.

Esterase associated resistance is not always associated with a reduced affinity for the natural substrate. Ozaki (1969) found that an increased ability to hydrolyse the substrate methyl butyrate by malathion-resistant *Nephotettix cincticeps* Uhler (green rice leafhopper) was correlated with an increased ability to hydrolyse the OP malathion *in vitro*. It was therefore concluded that resistance to malathion was associated with increased carboxylesterase activity hydrolysing the insecticide directly to malathion carboxylic acid. Similarly, Needham and Sawicki (1971) reported a difference in the ability of esterases derived from OP susceptible and resistant *Myzus persicae* (Sulzer) (the peach-potato aphid) to hydrolyse 1-naphthyl acetate, with high activity in OP resistant strains and a subsequent decrease in activity with reduced resistance. A method of separating the esterases of individual aphids by polyacrylamide gel electrophoresis (PAGE) was later devised by

Devonshire (1975), which revealed seven discrete esterase bands. The esterase that was found to have enhanced activity correlating with resistance was the fourth least mobile band and therefore named E4. Further studies on E4 using the OP paraoxon showed that activity in 1-naphthyl acetate assays and on PAGE gels was also responsible for insecticide hydrolysis. In addition, increased activity was shown to be a result of more E4 being present rather than a more active mutant form (Devonshire, 1977). Furthermore, the rate of esterase activity was shown to increase by the same factor between increasingly resistant aphid clones. This provided good evidence that the increase in esterase production was due to a 'succession of tandem duplications of the structural gene for E4' (Devonshire and Sawicki, 1979).

E4 was later shown to be responsible not only for organophosphate resistance but, as insecticidal spray use diversified, carbamate and pyrethroid resistance as well. Based on the equation:



where EH is the active esterase, AB is the inhibitor or substrate, EHAB is a Michaelis complex between the two and EA is acylated enzyme. The rate limiting step of the reaction, the hydrolysis of the acylated enzyme to regenerate free enzyme (k_3), was calculated to find the catalytic centre of activity of the *M. persicae* esterase for a range of inhibitors (Devonshire and Moores, 1982). From these studies, it was found that while the catalytic centre activity of E4 is very small, i.e. the turnover of insecticide per molecule is low, the large quantities produced by resistant aphids is sufficient to detoxify a significant dose of insecticide by both hydrolysis and sequestration.

In *Lucilia cuprina* (Wiedemann) (sheep blowfly) however, while resistance was found to also be caused by increased production of a carboxylesterase, similar enzyme re-activation studies showed detoxification of malaoxon and paraoxon to be caused by significant rates of hydrolysis (900 and 8000-fold faster respectively than E4 in *M. persicae*) rather than sequestration (Whyward and Walker, 1994).

1.2.1.1.2. *Glutathione-S-transferases*

Glutathione-S-transferases (GSTs) are soluble enzymes capable of catalysing the conjugation of nucleophilic substrates with an endogenous tripeptide glutathione (Soderlund, 1997). The effect of GSTs is to convert a reactive lipophilic molecule into a water-soluble, nonreactive conjugate which may be excreted (Vontas *et al.*, 2000). While it was proposed that elevated GST activity could result in resistance to both OPs and DDT (McKenzie, 1996), owing to the lack of specific GST inhibitors their role in insecticide resistance could not clearly be distinguished from other resistance mechanisms (Oppenoorth, 1985). Only with recent advances in purification techniques has direct evidence for their role in insecticide detoxification been provided (Soderlund, 1997). Such purification studies identified numerous different molecular structures of GSTs exhibiting different physical and catalytic characteristics (McKenzie, 1996). For example, two different groups of GSTs were identified in *M. domestica*, one with a high specificity for the model substrate CDNB (1-chloro-2,4-dinitrobenzene) over DCNB (1,2-dichloro-4-nitrobenzene), and another group with less substrate specificity (Clark *et al.*, 1984). It was later shown that insecticide degradation was associated with the latter group of GSTs (Clark *et al.*, 1986). This overexpression of GST activity, resulting in insecticide resistance, was later found to be caused by constitutive up-regulation of GST expression rather than duplication of the GST structural gene (Soderlund, 1997).

1.2.1.1.3. *Cytochrome P₄₅₀-dependent monooxygenases*

Cytochrome P₄₅₀-dependent monooxygenases or mixed function oxidases (MFOs) are involved in the detoxification of foreign compounds in insects and vertebrates (Soderlund, 1997). Numerous papers have reported that elevation in insect MFO activity results in resistance, primarily to carbamates, OPs and pyrethroids (McKenzie, 1996). However, there is little direct biochemical evidence supporting such claims. Soderlund and Bloomquist (1990) state that much of the work is based on the use of synergists, sub-lethal compounds designed to enhance the activity of an insecticide, without regard for the underlying biochemistry. Hence, piperonyl butoxide (PBO) is often used in synergism bioassays based on the assumption that it knocks out MFO activity. Even so, there is still debate over the mode of action of PBO and clear-cut conclusions about the role of MFOs based on these bioassays

alone cannot yet be made (Berge *et al.*, 1998). The synergist bioassays do, nevertheless, provide the first indication of a detoxification mechanism, which can then be tested for by biochemical assays.

1.2.1.2. Target-site alterations

1.2.1.2.1. Modified acetylcholine esterase

Acetylcholinesterase (AChE) is the target site of OPs and carbamates. Normally, the blocking of AChE by these inhibitors results in a build up of the neurotransmitter acetylcholine at nerve synapses resulting in nerve impulses being continually propagated leading to de-sensitisation. Insensitive AChE has an altered active-site that prevents binding by the insecticide and therefore allows synaptic transmissions to occur without disruption (Devonshire and Moores, 1984). The first report of this resistance mechanism was in a paraoxon-resistant strain of *Tetranychus urticae* Koch (two-spotted spider mite) (Smissaert, 1964). Resistance in this organism was associated with a reduced ability by the AChE to catalyse the hydrolysis of its natural substrate, acetylcholine. Whilst such a trait would be expected to confer a fitness cost, the protection from paraoxon provides an overall fitness advantage in the presence of the insecticide parathion. In addition, Smissaert *et al.* (1975) reported that insects possess a large excess of AChE and therefore, only a dramatic reduction in natural levels would confer any cost to the insect. Since this first report, insensitive forms of AChE have been found in numerous insect species. However, the altered target-site is not always associated with a reduced affinity for the natural substrate. Multiple insensitive forms of AChE have been identified in *M. domestica*, some of which demonstrated an increased affinity for acetylcholine i.e. a lower K_m . In addition, the actual rate of reaction (V_{max}) has been found to differ between AChE forms within this species (Devonshire and Moores, 1984).

The first insensitive AChE genes were sequenced in *Drosophila melanogaster* Meigen (fruit fly) (Fournier *et al.*, 1989) followed by *M. domestica* (Williamson *et al.*, 1992; Walsh *et al.*, 2001). In both species, combinations of mutations were needed to confer appreciable levels of resistance.

1.2.1.2.2. Knock-down resistance

The principal target-site in invertebrates of DDT and pyrethroids is the voltage-sensitive sodium channel (Soderlund and Bloomquist, 1990). Resistance to the rapid paralysing or 'knockdown' effect of these two compounds was first identified in the 1950s in association with DDT resistance in *M. domestica* (Busvine, 1951). This knock-down resistance (*kdr*) trait, occurring as a recessive allele, was later genetically isolated in *M. domestica* populations together with another resistance phenotype, *super-kdr* (*skdr*) which conferred even greater resistance to both insecticides (Farnham *et al.*, 1987). Unfortunately, unlike the previous resistance mechanisms discussed, there is no rapid biochemical test available for diagnosing *kdr*. Therefore, initial indications of *kdr* are inferred by diagnostic-dose bioassays (see 1.3.1) and systematic elimination of other resistance mechanisms. However, full confirmation of *kdr* resistance could previously only be achieved by radiolabelled insecticide metabolism studies (to rule out any metabolic resistance) and electrophysiological investigations of nerve sensitivity to the insecticides (to demonstrate conclusively a reduced sensitivity) (Soderlund and Bloomquist, 1990). Recent advances in molecular biology have now made characterisation of *kdr* easier by identification of the gene mutations involved. The most common of these causes a leucine to phenylalanine substitution in the domain IIS6 transmembrane region of the insect *para*-type sodium channel protein (Devonshire *et al.*, 1998). This was first identified in *M. domestica* (Williamson *et al.*, 1996) and *Blattella germanica* L. (German cockroach) (Miyazaki *et al.*, 1996) and later found in *M. persicae* (Martinez-Torres *et al.*, 1999) and several other insects.

The actual effect of the gene mutation was previously disputed (Soderlund and Bloomquist, 1990) although use of voltage clamp techniques have now revealed structural changes in the voltage-sensitive sodium channel, which alters the binding site of the insecticide (Vais *et al.*, 2001).

1.3. Methods of Monitoring Insecticide Resistance

The techniques available at present to monitor resistance can be divided into *in vivo* and *in vitro* assays (Ffrench-Constant and Roush, 1990). *In vivo* assays usually involve assessing the effects of insecticides on intact individuals. *In vitro* assays use biochemical techniques to assess enzyme activity or examine specific resistance genes. However, Ffrench-Constant and Roush (1990) suggest both techniques should

be used to complement each other rather than placing reliance on one single procedure.

1.3.1. Toxicological bioassays (*in vivo*)

Bioassays involve subjecting insects to specific concentrations of insecticide for a set period of time, after which the mortality of individuals can be assessed. Toxicological bioassays are divided into two types: dose-response assays and diagnostic dose assays. Dose-response assays involve exposing many individuals to a range of different insecticide concentrations that produce a 10-90% mortality range (Halliday and Burnham, 1990). From this information LD₅₀ or LD₉₀ values (the lethal dose at which 50% or 90% of the sample is killed) can be derived. This approach provides a good overview of resistance in a population where no previous work has been conducted. However, once an outline of resistance has been obtained for a population, this method is ‘a wasteful and inefficient use of resources’ (Denholm *et al.*, 1998), since low frequency resistance phenotypes, which can potentially have a huge effect on the future response of a population, will only be detected when large numbers of such individuals are present. As an alternative, the diagnostic dose assay uses one or very few specific insecticide concentrations to discriminate susceptible and resistant individuals (Figure 1.1). While this method allows many individuals to be tested quickly, it initially requires additional resistance information, such as dose-response data, in order to choose the most appropriate discriminating dose.

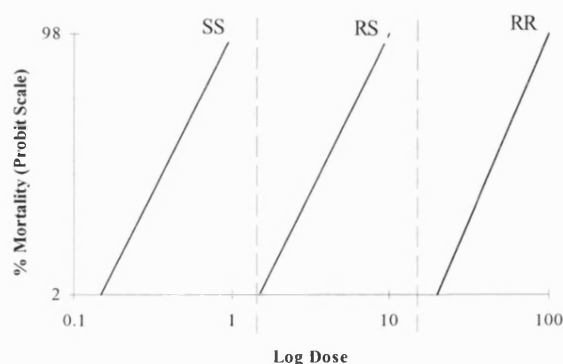


Figure 1.1 Representation of two diagnostic dose concentrations (dashed lines) which discriminate between all three genotypes. SS = homozygous susceptible, RS = heterozygous (which often show a degree of resistance) and RR = homozygous resistant. (From French-Constant and Roush, 1990)

Whichever assay method is chosen, an essential component of resistance monitoring is the choice of the standard or reference strain to which the response of field populations can be compared (Sawicki, 1987). Ideally, a standard strain should be free from insecticidal selection pressures. However, even in conditions that are free from insecticides, susceptibility of individuals to a particular insecticide within a population can vary enormously. One approach used by Forrester and Cahill (1987) to obtain a true susceptible response was to average the response of 34 susceptible field and laboratory strains. Alternatively, a susceptible field strain is used which shows the natural dynamic changes defined as a 'normal response' (Sawicki, 1987).

Despite such considerations for bioassay design, experiments often lack realism when compared with actual field conditions. Sawicki (1987) used the example of the mite slide dip bioassay where mites are restrained on sticky microscope slides, dipped into the appropriate concentration of insecticide and mortality is assessed within two days. When a population of *T. urticae*, thought to be resistant to the acaricide dicofol, were subjected to this treatment, they were found to be only around six times less susceptible than the standard susceptible strain (Dennehy *et al.*, 1983). When the same population was placed on leaves dipped in a field rate concentration of dicofol, the actual resistance level was seen to be over 500-fold. It is for this reason that bioassay data should ideally be supported by field experiments.

1.3.2. Biochemical assays (*in vitro*)

The major limitation of many of the toxicological bioassays is that the techniques only select at the phenotypic level (diagnostic dose techniques can be an exception as illustrated in Figure 1.1) which is adequate for general resistance monitoring but limits research with regards to changes in the underlying genetic structure of resistance (Denholm *et al.*, 1990). Biochemical assays can distinguish unambiguously between susceptible and resistant insects while reducing the sample size needed to detect resistance at a given level of probability (Roush and Miller, 1986). Biochemical assays include the detection of enzyme activity using model substrates, the use of enzyme-specific antisera to isolate specific enzymes, as well as the detection of specific DNA sequences (French-Constant and Roush, 1990).

The use of model substrates to measure levels of particular enzymes often provides a rapid way of screening field populations for resistance. Such assays exist for esterases, GSTs, MFOs and AChE. The biochemical assays are particularly useful for detecting subtle genetic aspects of resistance. For example, the specificity of the AChE assay has been used to discriminate *M. domestica* genotypes which overlap in response to toxicological bioassays (Denholm *et al.*, 1990). In addition, multiple alleles at resistance loci and multiple resistance mechanisms can be identified since homogenates of individual insects can be divided between several different assays involving different substrates and inhibitors (Devonshire and Moores, 1984). More recently, a fluorometric AChE assay adapted for resistance studies allowed single whiteflies (*Bemisia tabaci* (Gennadius)) to be assessed for AChE insensitivity. Subsequent bivariate plots showing responses of four different strains of *B. tabaci* to two different OPs showed a clear separation between strains (Figure 1.2) (Moores *et al.*, 2000).

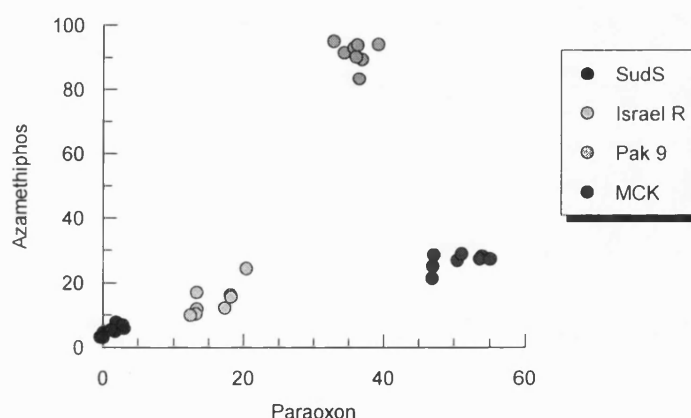


Figure 1.2 Bivariate plot of mean % activity remaining during inhibition of AChE from four strains of *Bemisia tabaci* by 10µM azamethiphos and 100µM paraoxon. Results were generated using a fluorometric AChE assay. (From Moores *et al.*, 2000).

Enzyme-specific antiserum techniques prove very powerful in high throughput resistance screening programs, for example the monitoring of the resistance related carboxylesterase, E4, in *M. persicae*. Production of antiserum is, however, costly in terms of time and resources and therefore generally only used in long term monitoring projects.

Molecular biology has provided more recent developments in *in vitro* techniques. The sequencing of resistance genes has the potential of providing methods by which specific DNA sequences can be used to probe for, and identify, resistance genes in populations of unknown genotype. At present, these techniques tend to be more costly and time consuming than biochemical methods and are therefore less applicable to current resistance management strategies. However, DNA alterations linked to resistance can now be identified and reveal resistance potential within populations of *M. persicae* (Field *et al.*, 1997) and new advances in the rapid diagnosis of single nucleotide changes will unquestionably increase the utility of this approach for monitoring resistance.

1.4. Insecticide Resistance Management

Insecticide resistance management (IRM) involves instigating a regime of various pest control methods and techniques on the basis of resistance monitoring data in order to 'extend the number of generations that a given pest population can be controlled economically by a pesticide' (Roush, 1989). The old methods of combating resistance, by increasing the amount of pesticide applied and replacing old chemicals with new, more effective chemicals, are no longer viable based on an improved knowledge of the cause of resistance and a decline in the rate of discovery of novel, effective compounds (Soderlund and Bloomquist, 1990).

1.4.1. Utilisation of monitoring data in IRM

Resistance monitoring should not only document resistance but also measure and identify resistant genotypes, provide early warning of impending resistance problems, determine changes in the distribution and severity of resistance and most importantly, provide information upon which a successful resistance management programme can be based and subsequently monitored (Brent, 1986). Unfortunately, in order for an IRM strategy to be effective, it has to be implemented as soon as resistance is discovered. It is this lack of specific quantitative information which makes applying bioassay results to an effective management strategy difficult (Roush, 1989). In addition, it is difficult to predict the future development of resistance with regard to migration and resistance genetics of a population. Even so, while the resistance characteristics of a particular population can always be

investigated further, it is often possible to design reasonable programmes on the basis of a relatively modest amount of information (Roush, 1989). Once a scheme has been implemented on the grounds of initial bioassay results, further, more detailed investigations may be conducted, such as diagnostic bioassays and biochemical analysis, in order to improve the scheme.

1.4.2. IRM strategies

Roush (1989) proposed that it is easier to examine the available management options (of which there are relatively few) and then implement a strategy rather than attempt to predict the development of resistance, which requires much more detailed information. For IRM to be successful, a distinction should be made between management of pesticide resistance (preventative IRM) and management of resistant pests (curative IRM) (Forrester, 1990). It is the preventative IRM strategies that are of most interest as these can be used before the evolution of resistance and will therefore potentially minimise crop losses.

Most tactics involve minimising the fitness of resistant genotypes either by preserving susceptible homozygotes or by killing resistant homozygotes and heterozygotes (Roush, 1989). Denholm *et al.* (1998) list the five key tactics implemented for preventative IRM as reducing the amount of insecticide applied, avoiding unnecessary persistence, creating refugia, alternating between unrelated compounds and employing strategic spray treatments against the most vulnerable life stages. Roush (1989) groups these strategies into preserving susceptible homozygotes and destroying resistant genotypes.

Preserving susceptible homozygotes includes reducing the amount of insecticide applied to a crop by less frequent applications or lowered application rates (Denholm and Rowland, 1992). This technique simply uses the knowledge that the lower the selection pressure, the slower a population will evolve resistance. Similarly, avoiding persistent compounds is also likely to reduce the selection pressure on pests. Creating untreated refugia in which susceptibles can proliferate is a less clear-cut technique which may compromise the quality of the crop being protected. Denholm and Rowland (1992) suggest that evidence which supports this strategy may be seen when the development of resistant pests in the field is compared with

that in glasshouses. For example, glasshouse populations of *M. persicae* developed resistance much faster than populations in the field because the former are subjected to the same treatment with less mixing of genes within the closed environment. This also highlights the difficulty of equating migration of pests in an open environment, disclosed by ecological techniques such as mark-release-recapture, with actual gene transfer.

Different life stages may also be treated as refuges for susceptible genes. By treating only particular life stages, susceptible genes may remain in the population. However, this may also compromise crop quality. Targeting a particular life stage can also be considered as a method to destroy resistant genotypes if certain stages are more susceptible to a compound than others (Roush, 1989). For example, *T. urticae*, has a high tolerance of mitochondrial electron transport inhibitors (METI) in the adult stage but is controlled well when nymphs are treated (Devine *et al.*, 2001).

Other key strategies for destroying resistant genotypes include high-dose strategies and insecticide mixtures. The high-dose strategy aims to kill at least all of the resistant heterozygotes in a population, although this method is only effective at the onset of resistance when resistance genes occur in the heterozygous state. If used at any other time it may simply speed up the development of resistance by exerting a strong selection pressure on a susceptible population or select for homozygotes in a resistant population.

Alternating between two unrelated compounds restricts the period for which a population is exposed to a given insecticide. Consequently, it can be considered a method that preserves susceptible individuals (those that are resistant to the compound in use but not to the other alternative). In some cases, alternations should also reduce the overall fitness of resistant individuals by continually changing the selection pressure. The mixture strategy involves using both compounds at once. The only survivors are those individuals that are resistant to both compounds. For this reason, as with the high dose strategy, it is important that some susceptible individuals are preserved to dilute the impact of these survivors (Denholm and Rowland, 1992). In this situation an untreated refuge may be a useful additional management tactic.

While other strategies have been proposed for IRM such as the use of synergists and spatial mosaics in which adjacent areas are treated with different chemicals (Denholm and Rowland, 1992), experience during the development of IRM has favoured the use of the tactics listed by Denholm *et al.* (1998) above. Equally importantly, a strategy must be environmentally and socially acceptable. Hence a high-dose strategy must be within the legal limits of human exposure and relatively easy to implement and unambiguous (Denholm and Rowland, 1992). In addition, resistance levels should continue to be monitored after the implementation of a strategy in order to provide justification for a grower to support a project (Forrester, 1990) as well as providing information to continue the project and identify the risks for the future.

1.5. Insecticide Resistance in *Nasonovia ribisnigri*

Aphids are of huge economic importance world wide in terms of loss of crops. They cause damage by direct feeding of phloem sap, virus spread and cosmetic damage. Their ability to reproduce parthenogenetically allows rapid colonisation of crops in optimal conditions and spread of advantageous genes, such as those conferring insecticide resistance.

The effective control of aphids on the foliage of outdoor lettuce is essential to ensure the marketability of this crop. In the UK, lettuce is colonised by a complex of aphids including the currant-lettuce aphid, *N. ribisnigri*, the peach-potato aphid, *M. persicae*, and the potato aphid, *Macrosiphum euphorbiae* (Thomas). *N. ribisnigri* is often the major pest and is much more specific to lettuce than the other two species, which attack several other crop and non-crop plants (Ellis *et al.*, 1995). On ecological grounds, *N. ribisnigri* is therefore a primary candidate for the selection of resistance to insecticides, which remain the mainstay of its control in the UK and elsewhere in Europe.

Published bioassay data of UK *N. ribisnigri* field populations showed widespread but varied levels of resistance to the carbamate, pirimicarb and lower, varied levels of resistance to pyrethroids and OPs (Barber *et al.*, 1999). Resistance was correlated with elevated esterase activity disclosed by polyacrylamide gel electrophoresis (PAGE) but no direct link was established. Bioassays conducted using field strains originating from southern France and Spain exhibited a maximum of 12-fold resistance at LC₅₀ to the OP acephate and 660-fold to the cyclodiene, endosulfan (Rufingier *et al.*, 1997). Maximum levels of resistance to the pyrethroid deltamethrin (28-fold) and the carbamate pirimicarb (19-fold) were intermediate to these extremes. Laboratory selection experiments using French field populations have since shown that endosulfan resistance can result from glutathione-S-transferase detoxification, and pirimicarb resistance from modified acetylcholinesterase (MACE) (Rufingier *et al.*, 1999).

1.6. Introduction to Current Work

This thesis describes the detailed characterisation of resistance in UK populations of *N. ribisnigri* based on the findings of the above literature review. Chapter two describes the process undertaken to identify resistant populations of *N. ribisnigri*. Chapter three discusses the characterisation of the mechanism of pirimicarb resistance while chapter four covers the purification of a resistance-associated esterase. Chapter five describes the implications of resistance in field control while chapter six brings together all of the findings and discusses them in the context of relevant literature.

2. IDENTIFICATION OF RESISTANCE

2.1. Introduction

In order to determine the resistance status of *N. ribisnigri* populations in the UK, growers were alerted to the current project and encouraged to send field samples to Rothamsted for resistance testing, particularly from areas suffering from control difficulties. The populations were mass reared and tested against the key insecticides used for lettuce control as identified by discussion with growers. The organophosphate, heptenophos, was briefly used at the beginning of the project but owing to the reduced use of this compound on lettuce it was removed from experiments. Conversely, as there was a possibility of special off label approval (SOLA) for pymetrozine during the course of the project, this compound was included in the bioassay testing at a later stage. In addition to bioassay screening, preliminary indications of resistance mechanisms were investigated using *in vitro* techniques for esterase, AChE and GST activity.

2.2. Materials and Methods

2.2.1. Aphid strains and rearing methods

Two laboratory ‘standard’ clones maintained in culture since their resistance characterisation by Barber *et al.*(1999) were: Nr1A, a clone derived from a susceptible strain initially established at HRI Wellesbourne in 1994 and transferred to Rothamsted in 1995; and Nr2A, a clone collected in 1997 from a site in Kent experiencing control problems with pirimicarb.

Samples received during the project (between 1999 and 2002) were screened for resistance as described below and either cloned, if of phenotypic interest, or discarded. A total of 12 populations were received from the north (five samples), south (two samples), east (four samples) and west (one sample) of England. Four of those populations (Nr4, Nr8, Nr10 and Nr12) were cloned on the basis of the bioassay responses described in 2.3.1.

All strains of *N. ribisnigri* were reared parthenogenetically in the laboratory on whole plants of *Lactuca sativa* cv. 'Webb's Wonderful', without exposure to insecticides, at 21°C with a 16:8h (L:D) photoperiod. Plants were changed regularly and new ones re-infested to avoid host plant deterioration and excessive crowding of aphids.

For biochemical comparison, frozen stocks of two reference clones of *M. persicae* originating from sugarbeet in the UK in 1974 and characterised at Rothamsted, were used. These were: US1L, a fully susceptible clone; and T1V, a clone exhibiting resistance to pirimicarb (5-fold), cypermethrin (65-fold) and OPs (up to 94-fold) (Sawicki and Rice, 1978). Resistance in T1V was later shown to be caused by overproduction of an esterase (E4) (Devonshire and Moores, 1982).

2.2.2. *In vivo* Screening

2.2.2.1. Insecticides

Formulated insecticides used for leaf-dip bioassays were cypermethrin ('Cythrin', 100g/l EC, Syngenta) (emulsifiable concentrate); deltamethrin ('Decis', 25g/l EC, Aventis); lambda-cyhalothrin ('Hallmark', 50g/l EC, Syngenta), pirimicarb ('Aphox', 500g/kg SG, Syngenta) (soluble granules) and pymetrozine ('Plenum', 250g/kgSG, Novartis). For leaf-dipping, all formulations were diluted to the required concentration in distilled water containing 0.01% 'Agral' (Syngenta), a non-ionic surfactant added to improve leaf-wetting and to compensate for the loss of formulant at low insecticide concentrations. Imidacloprid was applied topically as technical material (>99% purity; Promochem Ltd.) diluted to the required concentrations in acetone. Because pymetrozine was granted SOLA later in the project, some field populations received in the first and second year of the project were not tested with this compound.

2.2.2.2. Leaf-dip bioassays

Leaf discs (35mm diameter) cut from lettuce (*L. sativa* cv. 'Webb's Wonderful') were dipped in insecticide solution for 20s, placed upside down on an agar bed (25mm in depth) in disposable plastic containers (30mm high), and allowed to air-dry. Alate adult *N. ribisnigri* of the required strain (10 per container) were placed on

the treated leaf surface and confined by applying a ring of fluon to the exposed lip of the container. Leaf discs dipped in water plus Agral were used as controls. Bioassay containers were covered with a fine mesh lid and stored upright in a constant environment facility at 20°C under ambient daylight conditions.

2.2.2.3. Topical application bioassay

For bioassays with imidacloprid, alate adults were placed on untreated leaf-discs in containers (10 per container) as described above and dosed individually with a 0.25µl droplet of insecticide in acetone using a microapplicator and syringe (Burkard, Rickmansworth), with acetone alone used as a control. Treated aphids were stored as described above.

2.2.2.4. Design and analysis of bioassays

Dose-response bioassays against field populations were tested at least once over 3 - 5 concentrations with two batches of 10 alate adults per concentration. Owing to this low number of insects and the possibility of genetic heterogeneity within strains, no attempt was made to fit probit lines to these data. Adults incapable of co-ordinated movement of legs (after gentle prodding if necessary) were scored as dead. All bioassays were scored at intervals of 48h and 72h following initial exposure to insecticide. Populations which were subsequently cloned (Nr4A, 8A and 10A in addition to Nr1A and Nr2A) (see 2.2.1) were tested again against pirimicarb and cypermethrin using at least two batches of 10 alate aphids per concentration (i.e. 20 insects) at at least three insecticide concentrations. Repeated assays (between two and ten) against these clones were pooled for probit analysis using the POLO computer program (LeOra Software, Berkeley, California). The smallest pooled data set for cloned populations contained two replicates of three batches of 10 adults at six insecticide concentrations.

Associations between responses to pirimicarb and pyrethroids were investigated by pairwise comparisons of mortality data at concentrations yielding the widest range of responses to each individual insecticide (i.e. those of greatest diagnostic value).

2.2.3. Stability of esterases *in vivo*

To assess the stability of the esterase previously identified in *N. ribisnigri* (Barber *et al.*, 1999), four small lettuce plants in individual pots were infested each with 20 individuals of Nr2A derived from a population known to show high E0.34 activity on PAGE gels (see 2.2.4.2 and 2.3.2.1). The pots were covered with perforated clear plastic bags and left for eight weeks at 21°C with a 16:8h (L:D) photoperiod. During this time, no change to pot 1 was made. In pot 2, adult aphids were regularly removed to maintain a low population density (*c.* 10 individuals per plant). In pot 3, plant material was regularly changed and aphid numbers were kept low. In pot 4, only the plant material was changed, retaining as much of the aphid population as possible during the change over. After eight weeks, the remaining aphids from each pot were collected, frozen and later run on PAGE gels as described in 2.2.4.2.

2.2.4. *In vitro* Screening

2.2.4.1. Buffers

Throughout the biochemical studies, one of three buffers was normally used: 0.02M phosphate buffer, pH 7.0, containing 0.1% Triton X-100; 0.2M phosphate buffer, pH 6.0; or 0.02M 2-[N-morpholino] ethanesulfonic acid (MES), pH 6, containing 0.5% Triton X-100. These are referred to as pH7 PB, pH6 PB and pH6 MES respectively.

2.2.4.2. Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoretic patterns of non-specific esterases in individual aphids after homogenisation in sucrose/Triton X-100 (5%/1.6% w/v) were analysed using 7.5% polyacrylamide gel slabs containing 0.2% Triton X-100 and a discontinuous buffer system (Davis, 1964) run at 250V for 2h. Gels were rinsed in 0.2M phosphate buffer, pH 6.0 for 30min then stained in the same buffer containing 2.5mM Fast Blue RR and 30mM 1-naphthyl acetate in acetone to give a final concentration of 0.6mM. Gels were fixed and stored in 7% acetic acid.

2.2.4.3. Acetylcholinesterase (AChE) Assays

Assays characterising the sensitivity of AChE to pirimicarb were based on the methods of Moores *et al.* (1988) for rapid diagnosis of AChE sensitivity in individual insects from field populations. Single aphids (8 per field strain) were homogenised in 20µl phosphate/Triton buffer (pH 7.5, 0.1M phosphate containing 0.1% Triton X-

100) in separate wells of a 96-well microplate, and left for 30 min at 4°C to enhance AChE solubilisation. Buffer (100µl), homogenate (50µl) and 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) (50µl) were equilibrated in a fresh microplate, using duplicate samples of homogenate to give an uninhibited control synchronised with one inhibition reaction. Assays were started by the addition of acetylthiocholine iodide (ATChI) in buffer (100µl) with and without a diagnostic concentration of pirimicarb, to give a final substrate concentration of 0.5mM, a final DTNB concentration of 15µM and a final pirimicarb concentration of 10^{-5} M. Assays were monitored for 20 min by a Thermomax microplate reader (Molecular Devices), utilising SOFTmax software that subsequently fitted linear regressions to successive absorbance readings taken at a wavelength of 405nm from each well. The diagnostic concentration of pirimicarb was chosen, using a serial dilution of inhibitor, to give almost complete inhibition in Nr1A.

2.2.4.4. Glutathione S-transferase assays

Glutathione S-transferases (GSTs) were measured based on the methods of Habig *et al.* (1974). Single aphids were homogenised in 100µl pH 7.5 Tris/HCl buffer in individual wells of a 96-well microplate. 100µl of the indicator, reduced glutathione (GSH), was added with 100µl of the substrate, 1-chloro-2,4-dinitrobenzol (CDNB) or 3,4-dichloronitrobenzene (DCNB), to give final concentrations of 0.4mM indicator and 4mM substrate. Reactions were monitored for 20min at a wavelength of 340nm using a Molecular Devices Thermomax kinetic plate reader.

Protein content of the same individuals was assessed using the method of Bradford (1976). 5µl of the homogenate was put in a clean microplate to which 200µl Bradford reagent (Sigma) was added. The plate was incubated at 24°C for 5min after which a single endpoint reading was taken at 620nm using a Molecular Devices Thermomax kinetic plate reader. Serial dilutions of bovine serum albumin were used to create a standard curve from which actual protein content could be calculated.

2.2.4.5. *kdr* screening

Total RNA was extracted from ~ 50mg of adult Nr1A and Nr4A as described by Martinez-Torres *et al.* (1997). First strand cDNA was synthesised from total RNA using Superscript II reverse transcriptase and oligo dT primer (200ng). The cDNA

was used as template for two rounds of PCR using degenerate sodium channel primers (1° reaction with D1 and Dg2 primers; 2° reaction with D2 and D5). Primer sequences are shown in Table 2.1. Reaction conditions were as described by Martinez-Torres *et al.* (1997). The sodium channel fragments amplified were direct PCR sequenced using the internal aphid primer aph3. Sequence files were analysed using vector NTI and Wisconsin GCG software packages. All primers were bought commercially from MWG-Biotech.

Table 2.1 Oligonucleotide primers (R=A+G, Y=C+T, D=A+G+T, W=A+T, S=G+C, M=A+C, N=A+C+G+T)

Name	Sequence
D1	AARYTNGCNAARTCNTGGCC
Dg2	GCDATYTTTRTTNGTNTCRTTRTC
D5	GCNAARTCNTGGCCNAC
aph3	TTGGTTCTCCGACGTG

2.3. Results

2.3.1. *In vivo* screening

By pooling the bioassay results for each of the six standard clones of *N. ribisnigri*, a uniform response was obtained for each individual clone that demonstrated general trends seen in field strains over the course of the three year project. Figure 2.1a shows a clear shift to the right in response, i.e. resistance, to cypermethrin by Nr4A, Nr10A and Nr12A, which was supported by probit analysis (Table 2.2). Together, these results showed that Nr4A and Nr12A exhibited high levels (20- to 25-fold) of resistance while Nr10A demonstrated an intermediate response of around 11-fold resistance to cypermethrin. The other three populations, Nr1A, Nr2A and Nr8A, were regarded as fully susceptible to cypermethrin.

The responses to pirimicarb (Figure 2.1b) were less clear-cut but there was still a significant shift in response to the right by Nr2A and slightly by Nr4A. In terms of

probit analysis, however, this represented only a 3.5-fold and 1.8-fold difference from Nr1A, respectively (Table 2.2).

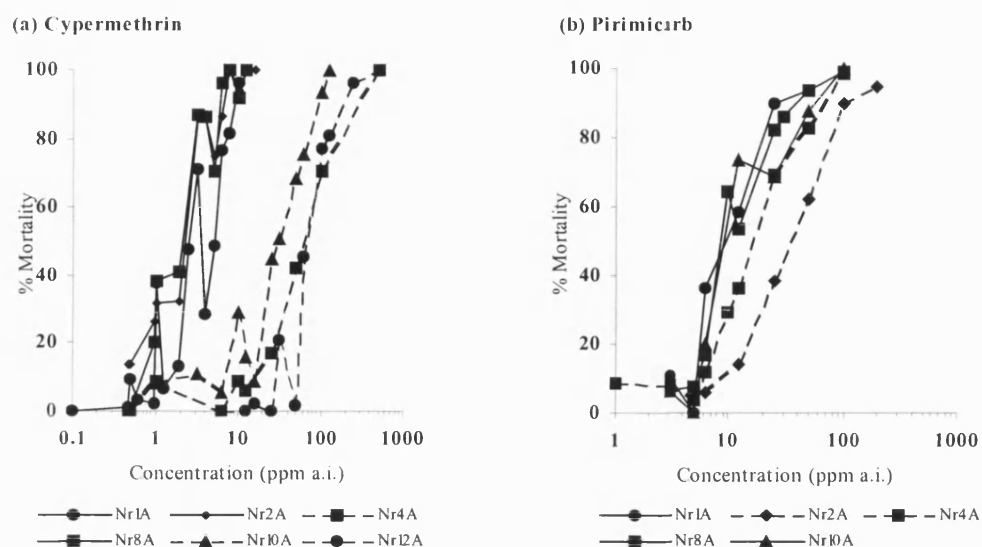


Figure 2.1 Pooled bioassay data (at least two sets) for the clones used throughout the study against (a) cypermethrin and (b) pirimicarb. Solid and dashed lines have been used to discriminate between those populations considered susceptible and resistant respectively.

Table 2.2 Probit analysis of pooled bioassay responses for the six clones of *N. ribisnigri*

	Cypermethrin				Pirimicarb			
	LC50 ¹	CL ²	Slope	RF ³	LC50 ¹	CL ²	Slope	RF ³
Nr1A	2.92	1.8-4.1	2.0		9.78	8.2-11.4	2.6	
Nr2A	1.72	1.2-2.3	2.3	0.6	34.44	27.0-41.9	2.4	3.5
Nr4A	59.87	45.8-73.1	2.9	20.5	17.60	14.5-20.7	2.5	1.8
Nr8A	1.88	1.4-2.4	2.6	0.6	11.91	10.0-13.9	2.7	1.2
Nr10A	32.07	23.3-39.8	2.7	11.0	14.74	9.8-19.6	2.6	1.5
Nr12A	74.99	61.5-88.4	4.0	25.7				

¹ Lethal concentration resulting in 50% mortality. Expressed as ppm active ingredient.

² 95% confidence limits for fitted LC₅₀ values.

³ Resistance factor compared to the standard susceptible, Nr1A.

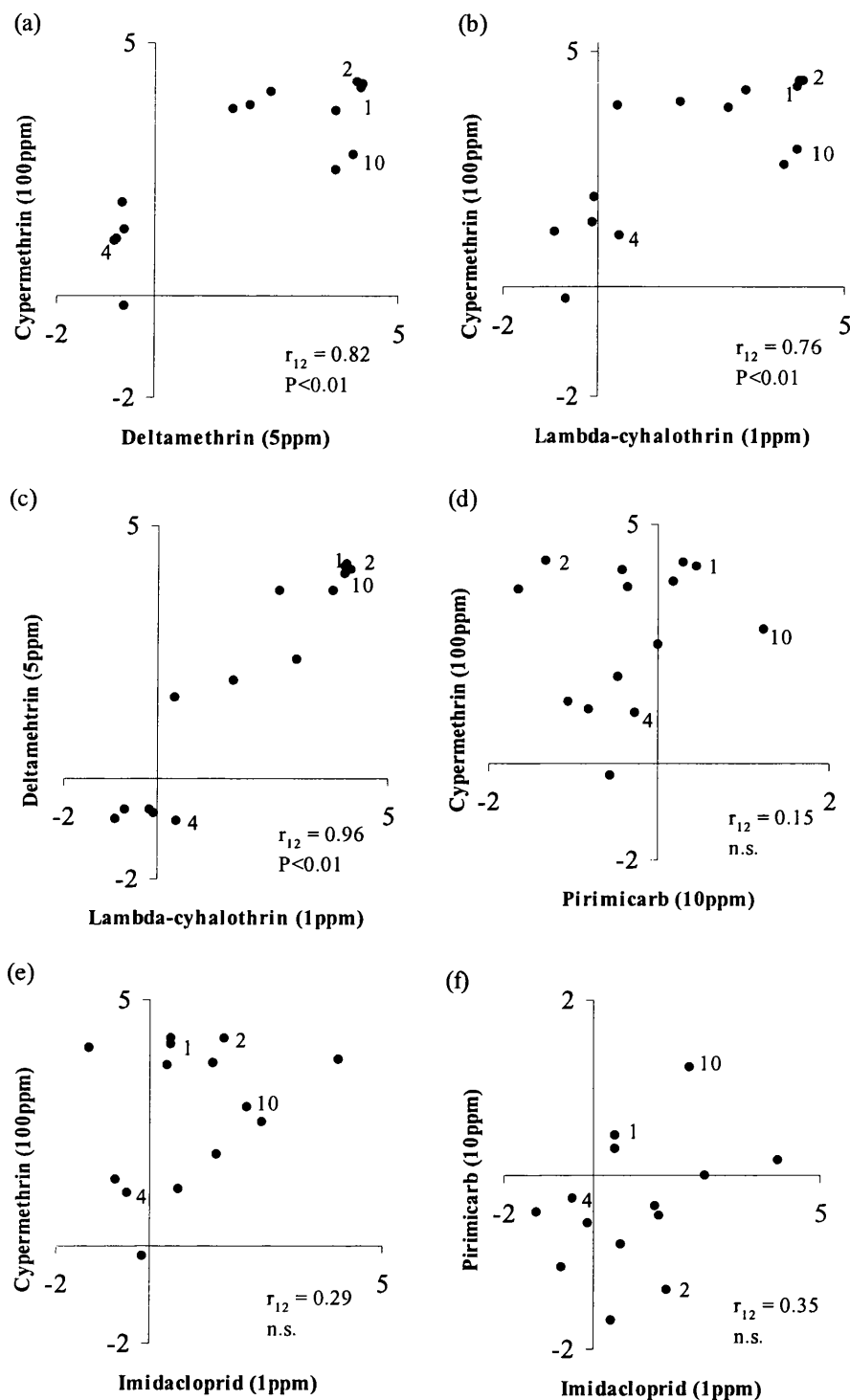


Figure 2.2a-f Bi-variate plots showing responses of 14 *N. ribisnigri* populations (including six cloned populations) to a single dose of five different insecticides after 72h. Axes show logit transformed mortality data. There was a significant positive correlation between all three pyrethroids tested ($P < 0.01$). n.s. = not significant. Numbers indicate responses of four clones, Nr1A (S), Nr2A (pirimicarb R), Nr4A (pyrethroid R) and Nr10A (intermediate R).

Leaf-dip bioassays showed a wide range of resistance levels by field populations to all five compounds tested. However, Figures 2.2a-c show a close association of responses (i.e. cross-resistance) to all three pyrethroids, whereas Figure 2.2d shows no correlation between pyrethroid and pirimicarb resistance. In addition, there was no correlation between responses to imidacloprid and either of the other two classes of compounds (Figures 2.2e-f). $P < 0.01$.

Figure 2.3a shows the responses of the field populations collected during 2001 to imidacloprid compared with the response of Nr1A. Of the fourteen populations tested between 1999 and 2001 (not all data shown), only two populations (Nr25 and Nr26) were more tolerant to the compound than Nr1A and all populations were killed by 10 ppm.

Results for pymetrozine varied between populations, with all clones being less susceptible than Nr1A (Figure 2.3b).

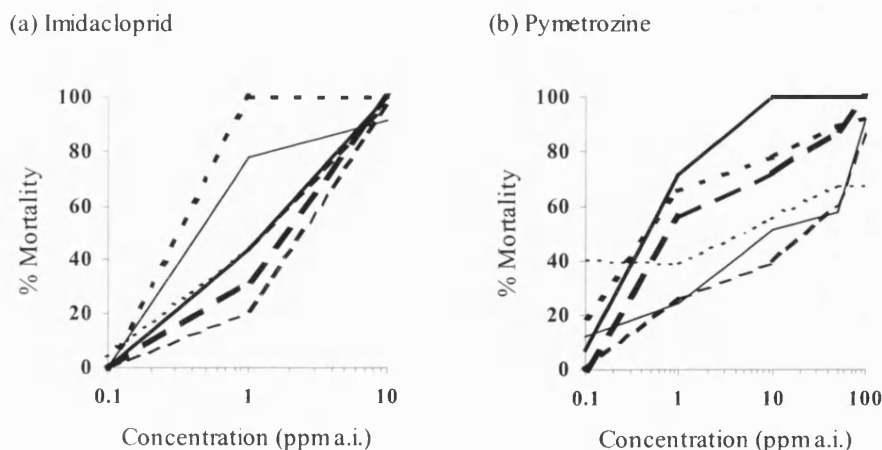


Figure 2.3a-b Responses of *N. ribisnigri* populations received in 2001 to (a) imidacloprid and (b) pymetrozine after 72hrs. Results include the pirimicarb susceptible and resistant clones Nr1A and Nr2A.

Nr1A (—), Nr2A (•••••), Nr25 (— —), Nr26 (— — —), Nr28 (——) and Nr29 (— · — · —)

2.3.2. *In vitro* screening

2.3.2.1. Esterase analysis

In keeping with previous results (Barber *et al.*, 1999), when stained for activity using 1-naphthyl acetate, PAGE gels disclosed an enhanced esterase band, E0.34 (named according to its relative mobility on the gel), in pirimicarb resistant (R) *N. ribisnigri* individuals (Nr2A) compared with the laboratory susceptible (S) clone (Nr1A) (Figure 2.4). Similar observations have previously been reported in amounts of esterase E4 in S (US1L) and R (T1V) *M. persicae*. However, the *N. ribisnigri* resistance associated esterase was clearly different from that in *M. persicae* in terms of relative electrophoretic mobility.

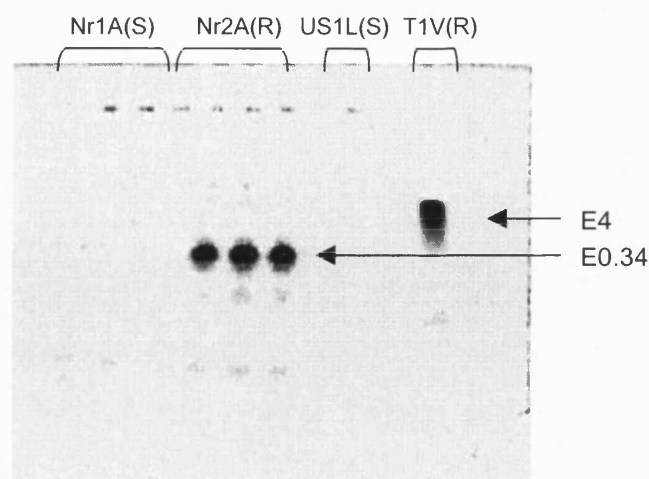


Figure 2.4 Esterase banding patterns in pirimicarb susceptible (S) and resistant (R) clones of *N. ribisnigri* (Nr1A and Nr2A) and *M. persicae* (US1L and T1V).

Field strains exhibited a variety of stain intensities on gels when treated with 1-naphthyl acetate. Comparison with the six clones shows a similar mix of high, medium and low intensity staining (Figure 2.5). The increase in E0.34 band intensity was found to correlate well with the reduced mortality levels of field populations at a diagnostic concentration of pirimicarb (10ppm) (Figure 2.6).

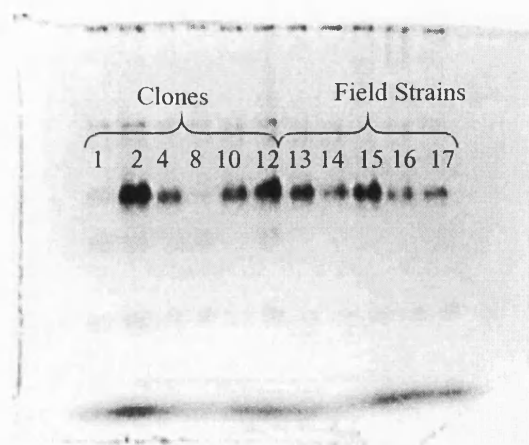


Figure 2.5 Esterase banding patterns in six clones (Nr1A, 2A, 4A, 8A, 10A, 12A) and five field strains (Nr13, 14, 15, 16, 17) of *N. ribisnigri*.

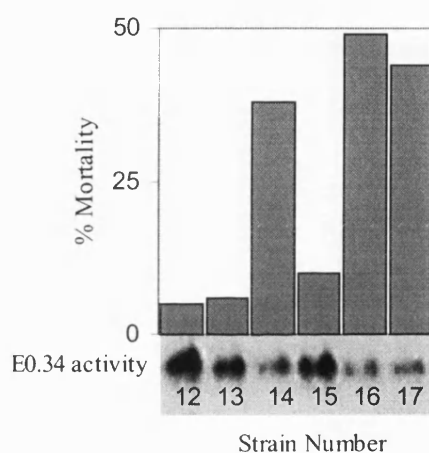


Figure 2.6 Comparison of E0.34 stain intensity in *N. ribisnigri* field populations and response to a single concentration (10ppm) of pirimicarb after 72hrs exposure.

When rearing pirimicarb resistant *N. ribisnigri* clones for bioassays, it was noted that the enhanced esterase band associated with resistance was becoming fainter. This change on the PAGE gels correlated with a decrease in resistance in pirimicarb bioassays while responses to pyrethroids remained constant in all populations (Figure 2.7). By subjecting individuals of Nr2A, known to have the enhanced band, to different population densities and plant qualities as outlined in Figure 2.8, the decreased expression of the esterase was associated with overcrowded rearing conditions (conditions A and D on Figure 2.8).

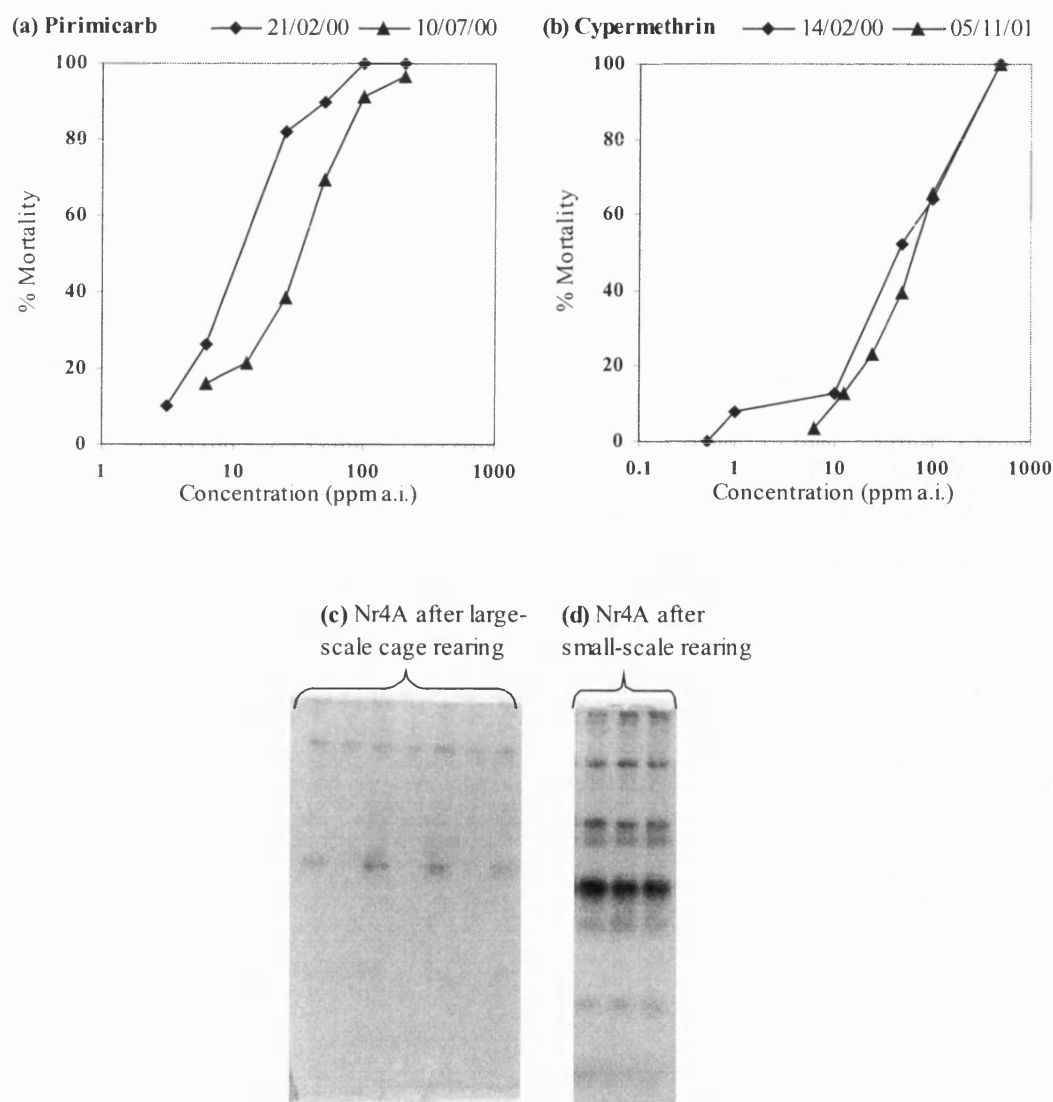


Figure 2.7 Comparison of responses by Nr4A individuals to (a) pirimicarb and (b) cypermethrin at two different dates. The first date (14th or 21st February 2000) represents a period where E0.34 activity was low in populations reared in cages (c) compared to esterase levels in individuals from small, low density rearing (d) represented at the later bioassay date.

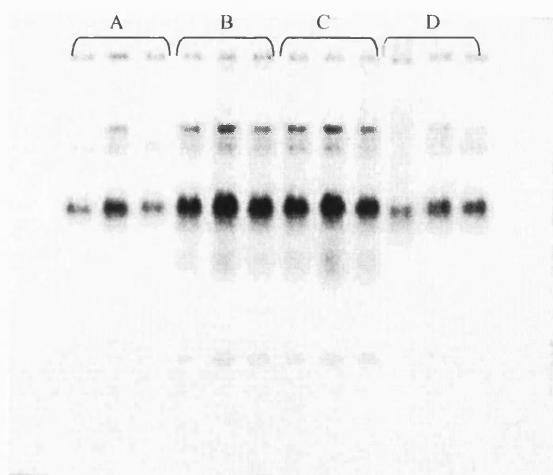


Figure 2.8 Individual Nr2A aphids on a PAGE gel after four weeks in one of the following conditions: (A) No change in plant material and increasing population density; (B) Low population density maintained and no change in plant material; (C) Regular change of plant material and low population density maintained; (D) Regular change of plant material and increasing population density.

2.3.2.2. AChE Screening

Throughout the project, a discriminating concentration of 10^{-5} M pirimicarb disclosed no evidence of MACE-type resistance to pirimicarb in any of the populations i.e. AChE levels were all uniformly reduced to significantly low levels with no difference within or between populations (Figure 2.9).

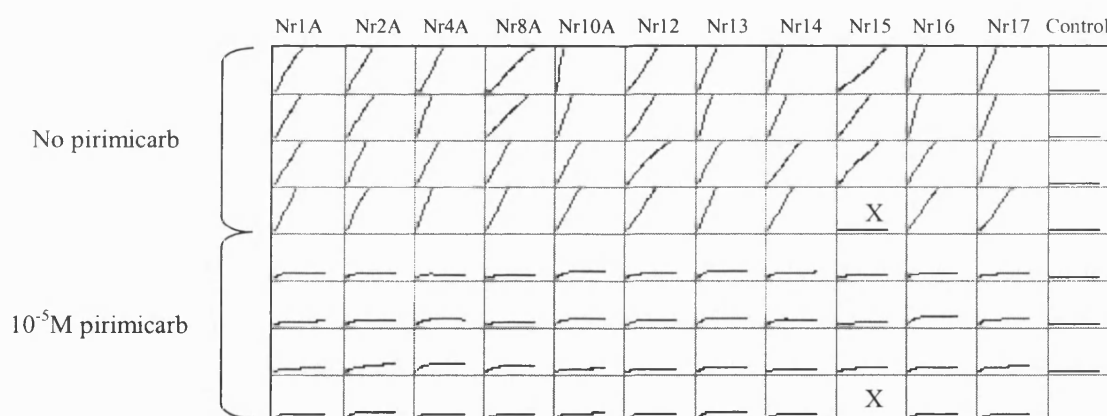


Figure 2.9 Kinetic plots for an AChE insensitivity assay in a 96-well microtitre plate. Four individuals of each strain were plated in the first four rows. Half of each aphid homogenate was transferred to the next four rows and incubated with 10^{-5} pirimicarb before starting the reaction. Controls and those wells marked with an X contained no aphid homogenate.

2.3.2.3. GST Assays

Reactions with the substrate DCNB were not sensitive enough to distinguish responses by single aphids. The substrate CDNB was readily broken down in the presence of GSH and aphid homogenate (Figure 2.10) and significant differences were seen between the mean values of some populations (Table 2.3). After accounting for protein content, Nr12A, 13, 15 and 29 were found to have the highest GST activity (*c.* 270mOD⁻¹mg⁻¹ml⁻¹ of protein) compared with Nr4A (*c.*150mOD⁻¹mg⁻¹ml⁻¹ of protein) and the susceptible clone, Nr1A (200mOD⁻¹mg⁻¹ml⁻¹) (Figure 2.11).

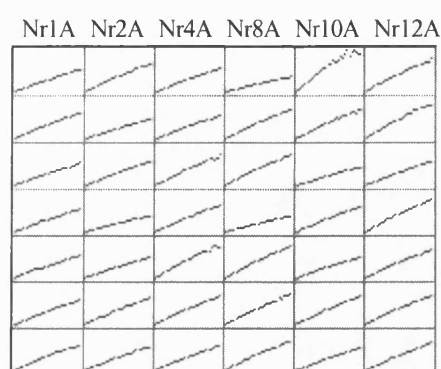


Figure 2.10 Kinetic plots for a GST activity assay in a 96-well microplate using the substrate CDNB.

Each well contains a single aphid. Slopes represent activity in mODmin⁻¹.

Table 2.3 Mean GST activity and standard errors (STE) in six clones accounting for control activity (i.e. activity observed in the absence of homogenate = 39.5 ± 0.4). Units = mODmin⁻¹.

	Nr1A	Nr2A	Nr4A	Nr8A	Nr10A	Nr12A
Mean	45.98	44.01	59.84	56.07	57.52	68.90
STE	2.48	5.36	4.93	9.88	9.56	4.79

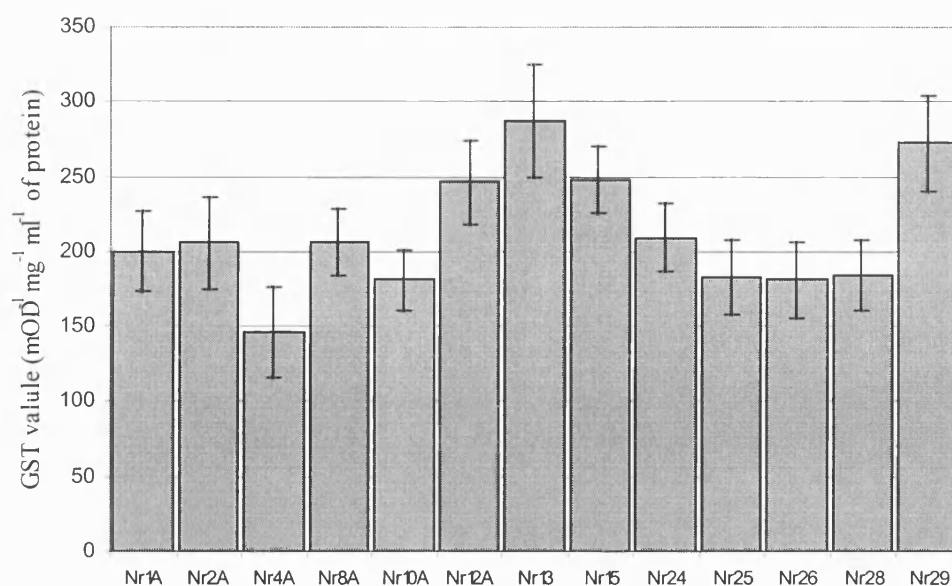


Figure 2.11 Mean (seven individuals) GST activity in the presence of CDNB accounting for protein content.

2.3.2.4. *kdr* screening

No genetic difference was found between the pyrethroid susceptible clone, Nr1A, and pyrethroid resistant clone, Nr4A, at the mutation sites within the domain IIS6 transmembrane region of the sodium channel, known to cause *kdr* and *skdr* in *M. persicae*.

2.4. Discussion

The monitoring of UK field populations of *N. ribisnigri* in 1999 at the beginning of the project identified six populations which exhibited one of four phenotypic modes: (1) susceptible to all insecticides tested (Nr1A and 8A); (2) resistant to pirimicarb (Nr2A); (3) resistant to pyrethroids (Nr10A and 12A); (4) resistant to pirimicarb and pyrethroids (Nr4A). These populations were cloned and formed the standards by which other field populations were compared in both *in-vivo* and *in-vitro* work.

When UK populations of *N. ribisnigri* were first investigated for insecticide resistance, mode 2 resistance was common in the populations studied and, in Nr2A, conferred up to 10-fold resistance to pirimicarb (Barber *et al.*, 1999). The occurrence of pirimicarb resistance was associated with an elevated esterase, named E0.34 based on its relative mobility on a PAGE gel. This correlation was identified

in the present work, however, the LC50 value for pirimicarb of the mode 2/4 (pirimicarb resistant) populations appeared to decline. Pooling the pirimicarb bioassay results for Nr2A, collected during the three year project, resulted in an RF of only 3.5. However, low-level pirimicarb resistance was shown to confer significant control difficulties in the field (see chapter 5).

Elevated E0.34 appeared to be present in aphids exhibiting mode 3 resistance as well as 2 and 4 i.e. elevated E0.34 appeared to correlate with pyrethroid resistance as well. However, by observing the natural loss of E0.34 under overcrowded rearing conditions with the simultaneous reduction in pirimicarb resistance, without any change in pyrethroid resistance status (in Nr4A), it was clear that two different resistance mechanisms were present in UK populations of *N. ribisnigri*.

The spontaneous loss of the enhanced esterase band in some populations, and the resulting loss of pirimicarb resistance, provided circumstantial evidence for the involvement of the elevated esterase in pirimicarb resistance. The occurrence of such 'revertants' is well documented in *M. persicae* and is known to be caused by the 'turning off' of esterase gene copies. However, the trigger for this phenomenon is not yet known (Devonshire *et al.*, 1999). In the case of *N. ribisnigri*, the rapid loss of the band (within four weeks when subjected to increasingly crowded conditions) and the rapid recovery of the band (c. 4 weeks in optimal rearing conditions) suggests that the basis for reversion may also be caused by direct chemical inhibition of the esterase rather than a genetic change. Such a chemical could perhaps be produced by the lettuce plant as a result of feeding damage or even by the aphids themselves in response to over crowding. It is more likely, however, that it is caused by a redistribution of resources by the aphid as a response to starvation. To ensure this phenomenon did not affect subsequent bioassays and biochemical studies, populations were regular re-seeded on new plants.

While pirimicarb-resistant populations appeared to decline in frequency and intensity during the course of the project, pyrethroid resistance increased, with a maximum RF value of 26 to cypermethrin compared with that reported by Barber (1999) of 10-fold resistance. Populations resistant to one pyrethroid exhibited cross-resistance to the other two pyrethroids tested. One likely cause of this high resistance, *kdr*, was not

found by screening for the same sodium channel mutations as those found in *M. persicae* and many other insect species. However, it is possible that other mutations exist in a different region of the sodium channel.

No MACE mechanism was identified in any of the populations, although from experience with *M. persicae*, much greater levels of pirimicarb resistance would be expected if this mechanism was present in UK populations.

Screening for GST activity did identify differences between populations. However, these differences could not be correlated to any particular mode of resistance. For example, the pyrethroid resistant Nr4A exhibited the lowest activity while the susceptible Nr1A and pirimicarb resistant 2A had equal activity. Another pyrethroid resistant clone, Nr12A, exhibited the highest activity.

The majority of bioassay responses to imidacloprid demonstrated full susceptibility to the compound compared to Nr1A. Although it is probable that the shift in response by two populations tested in 2001 would not cause a direct resistance problem in the field, low level tolerance to the compound can result in significant survival on parts of a plant with a reduced concentration of imidacloprid (Foster *et al.*, In Press). As imidacloprid is applied on lettuce as a seed treatment, the dose is reduced as the plant grows and the compound is dispersed systemically. With the decline of organophosphate use and limited choice of alternative chemistry, lettuce growers have become reliant on imidacloprid for chemical control of *N. ribisnigri*, particularly at the beginning of the growing season. Therefore, the potential risk of imidacloprid tolerance/resistance should continue to be monitored.

Although pymetrozine has only recently been approved for SOLA use on lettuce (HDC News, 2002), bioassay results showed variable performance against the adult *N. ribisnigri* populations tested. However, the results represent only preliminary data for this new compound and previous work using *M. persicae* (Foster *et al.*, 2002) suggests that, owing to its anti-feedant activity, more effective and accurate results would be obtained by testing the compound against nymphs rather than adults.

When considering the results of the bioassay analysis, it must be remembered that only a very small sample is represented of the different populations that were received at random from growers and advisors. The decline in the occurrence and intensity of pirimicarb resistance may have simply been a result of growers being able to control those populations based on the advice distributed in years 1 and 2 of the project (for example Barber *et al.*, 2001; Kift *et al.*, 2002). Pyrethroid resistance, however, may have proved more difficult to control and therefore samples were collected and sent for analysis. In addition, the relationship between bioassays and field performance can be complex, as described by Sawicki (1987). Therefore field experiments were designed and adapted throughout the project in order to confirm or refute the conclusions based on laboratory work (see chapter 5).

The *in-vivo* and *in-vitro* screening of *N. ribisnigri* populations showed:

- Two independent resistance mechanisms conferred pirimicarb and pyrethroid resistance in UK *N. ribisnigri* populations.
- Pirimicarb resistance was correlated with an elevated esterase, E0.34.
- Elevated esterase activity was reduced/lost in overcrowded rearing conditions.
- No *kdr/skdr* mutation was found in the same location of the sodium channel as that of *M. persicae*.
- From the samples received, the frequency and intensity of pirimicarb resistance declined over time while pyrethroid resistance became more significant.
- Modified AChE was not involved in the identified resistance and no correlation was found with elevated GST activity using the substrate CDNB.
- All but two populations proved equally or more susceptible to imidacloprid than Nr1A.
- Of the populations tested, responses were varied in pymetrozine bioassays against adult *N. ribisnigri*.

3. INVESTIGATION OF AN ESTERASE ASSOCIATED WITH PIRIMICARB RESISTANCE

3.1. Introduction

Resistance screening allowed field populations to be classed broadly into four different resistance categories: (1) fully susceptible; (2) pirimicarb resistant; (3) pirimicarb and pyrethroid resistant and (4) pyrethroid resistant. These distinctions between populations provided the first evidence that two different resistance mechanisms were occurring independently, one to pirimicarb and one to pyrethroids.

Pirimicarb resistance had been associated with an elevated esterase, E0.34, using PAGE (Barber *et al.*, 1999) but in order to screen large field populations rapidly for pirimicarb resistance, a diagnostic assay was required. It is known that esterases attack the ester bond present in pirimicarb (Figure 3.1). The total esterase assay described in 1.2.1.1.1, utilises 1-naphthyl acetate as a substrate, which also contains an ester bond but does not inhibit esterase activity. In the presence of water, 1-naphthol is produced (Figure 3.2) which reacts with the diazonium salt present in the reaction mix, to form a coloured complex, the intensity of which is a measure of esterase activity.

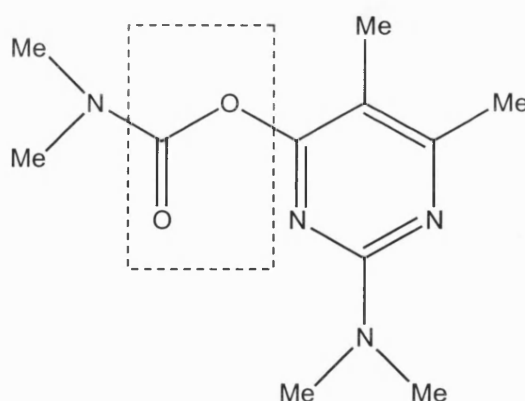


Figure 3.1 The structure of pirimicarb. The dashed box outlines the ester region, the target of esterases.

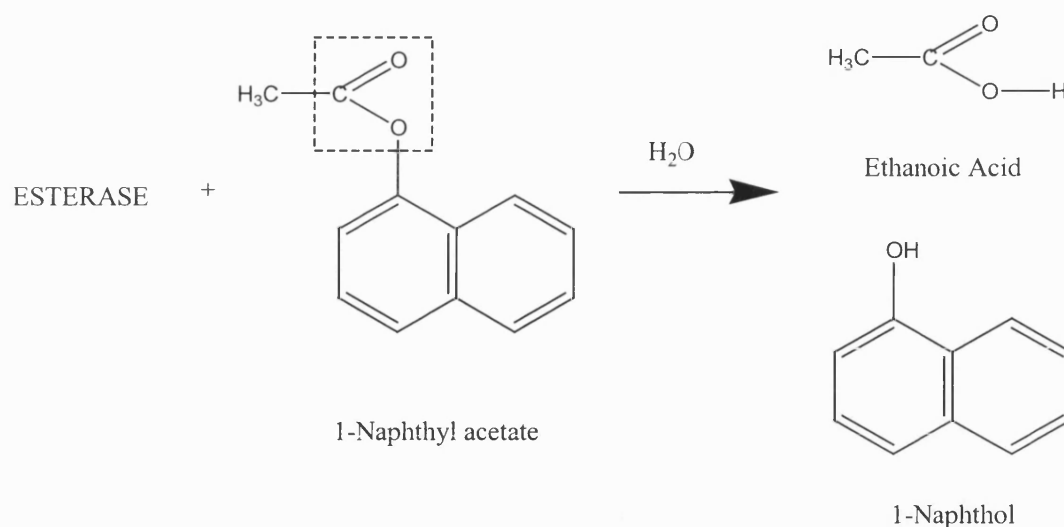


Figure 3.2 The total esterase assay reaction. The dashed box outlines the ester region hydrolysed by esterases. The free naphthol reacts with a diazonium salt to produce a measurable colour.

3.2. Materials and Methods

3.2.1. Total esterase assays

Total esterase assays, used to distinguish individual aphids rapidly on the basis of esterase activity, were initially based on the methods of Grant *et al.* (1989). Single aphids (Nr1A or Nr2A) were homogenised in 20µl pH 7 PB in individual wells of a 96-well microplate. A separate sample of pH 6 PB containing 0.75mM Fast Blue RR salt was filtered, and 1-naphthyl acetate in acetone added to give a final substrate concentration of 1mM. 200µl of this was added to each homogenate and mixed. Reactions were monitored for 10 min at 450nm using a Molecular Devices Thermomax kinetic plate reader.

After initial trials of the total esterase assays, variables were adjusted in order to optimise the method for discriminating between high and low esterase individuals.

3.2.1.1. pH

To determine the optimum pH for the total esterase assay, four samples of 0.02M MES, N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) and tris HCl buffer were adjusted respectively to pH 6.0, 7.0, 8.0 and 9.0 by the addition of 1M NaOH. The buffered solutions replaced the pH7 PB and pH6 PB normally used

in the total esterase assay. Single Nr2A alate adults were homogenised in 20µl of one of the buffers (eight replicates per pH treatment) and 200µl substrate, made with the same pH buffer, was added. Reactions were monitored as described above.

3.2.1.2. Homogenate concentration

The effect of aphid homogenate concentration in each well was assessed using a serial dilution of homogenate. 15 aphids of either Nr1A or Nr2A were homogenised in 150µl pH7 PB, centrifuged at 1100 g for 10 s and the supernatant taken. 20, 18, 15, 12, 10, 5, 2 and 0µl of supernatant was added to individual wells of a 96-well microplate. Volumes were made up to 20µl using pH 7 PB. 10µl was equivalent to one aphid. Assays were initiated by the addition of substrate and the reactions monitored as described above.

3.2.1.3. Total protein concentration

The effect of aphid size on total esterase content was assessed by measuring both the esterase and protein levels of individuals. 48 single Nr1A and Nr2A aphids were homogenised in 25µl pH 7 PB in individual wells of a 96-well microplate. 15µl of each homogenate was transferred to a clean microplate and assessed for total esterase activity using the 10 minute kinetic assay described above. Protein content was assessed using the method described in 2.2.4.4.

3.2.1.4. Assay run time

Eleven Nr1A and Nr2A aphids were homogenised individually in a microplate and assessed for total esterase content as described above, however, the assays were continued for 2hrs. This was repeated using 48 individuals of both clones, running the assay for 3hrs. Finally, six individuals from twelve different populations (the clones Nr1A, 2A, 4A, 10A and 12A and the mixed populations Nr13, 15, 24, 25, 26, 28, 29) were assayed for 3hrs.

3.2.1.5. Role of AChE

To establish the proportion of esterase activity contributed by AChE in the total esterase assay, the AChE inhibitor, eserine, was used. Twenty individuals of Nr1A or Nr2A were homogenised in 200µl pH7 PB, centrifuged at 1100 g for 10 s and the supernatant taken. 10µl 10⁻⁵M eserine was added to 100µl of supernatant. All

samples were incubated for 30mins at 24°C, at which point 20µl of both inhibited and uninhibited homogenates was taken and assayed for total esterase activity using the 10 minute method described above. In addition, to ensure the AChE activity had been inhibited by the eserine, 50µl of each sample was assessed for AChE activity using the method described in 2.2.4.3.

3.2.2. Esterase stability *in vitro*

The stability of E0.34 over time was assessed both electrophoretically and as part of the total esterase component of the aphid. 0.3g of Nr2A was homogenised in pH6 MES buffer. Immediately after homogenisation, a 50µl sample was taken and frozen. The mass homogenate was centrifuged at 1500 g for 5mins and the supernatant taken, at which point another 50µl sub-sample was collected (ten minutes after homogenisation) and immediately frozen. The remaining mass homogenate was incubated at 24°C. Further 50µl sub-samples were removed and frozen at 45mins, 75mins, 135mins, 255mins, 370mins, 435mins, 455mins and 1380mins after homogenisation. A final sample was taken at 1410mins but not frozen to act as control. After defrosting, 10µl of each sample was added to 10µl sucrose/Triton X-100 solution (5%/1.6% w/v), 15µl of which was loaded onto a PAGE gel and run as described in 2.2.4.2. 10µl of the defrosted samples were added to a 96-well microplate and assessed for total esterase activity as described above.

3.2.3. Effect of pirimicarb on esterase activity

The effect of pirimicarb on esterases in Nr2A homogenate was compared with the *M. persicae* clone, T1V in both PAGE analysis and by total esterase assessment. 30 aphids of Nr2A or T1V were homogenised in 200µl pH 7 PB, centrifuged at 1100 g for 10 s and the supernatant taken. Two 50µl samples were taken from both homogenates. Samples were incubated with and without 2µl 10⁻¹M pirimicarb (technical) in acetone at 24°C for 30mins. 15µl of each sample was loaded onto a five channel PAGE gel and run as described in 2.2.4.2. 20µl of the remaining samples were taken at 30, 90 and 180mins after the addition of pirimicarb and tested for total esterase activity as described above. In addition, 50 aphids of Nr1A and Nr4A were homogenised in 500µl pH 7 PB, centrifuged at 1100 g for 10 s and the supernatant taken. Two 250µl samples were taken from both homogenates. Samples

were incubated with and without 7.5µl 10⁻¹M pirimicarb at 24°C for 30mins after which 20µl of each sample was tested for total esterase activity as described above.

3.2.4. Radiolabelling

In order to measure possible quantitative differences in esterases between Nr1A and Nr2A, esterases were radiolabeled before running on a PAGE gel. 10 aphids of clones Nr1A and Nr2A were homogenised in 25µl sucrose/Triton X-100 (5%/1.6% w/v). The mass homogenate was centrifuged at 1100 g for 10 s and the supernatant taken. 2µl ³H diisopropylfluorophosphate (DFP) (310 GBq/mmol) was added to 15µl of each supernatant and centrifuged again at 1100g for 10 s. After a 30 min incubation at 24°C, 10µl of each supernatant was run on a PAGE gel, as described in 2.2.4.2, together with 10µl of the remaining uninhibited homogenates. After rinsing in distilled water for 30 min, the gel was soaked in 1M sodium salicylate solution for 30 min. The gel was then dried for 3hrs and placed next to X-ray film (Fuji medical X-ray film) and stored at -80°C for 12 weeks prior to development.

3.3. Results

3.3.1. Total esterase assays

Levels of esterase activity using the substrate 1-naphthyl acetate in Nr1A and Nr2A did not differ sufficiently to provide an accurate diagnostic technique for screening field populations for insecticide resistance (Figure 3.3). The main overlap in response between S and R individuals was within the range of 13 to 27 mOD min⁻¹ but in addition, some individuals of Nr1A exhibited even greater responses, thus overlapping with even the high Nr2A values. A PAGE gel, run as part of the continual monitoring of clonal integrity, disclosed several other esterase bands present in both Nr1 and Nr2A. The intensity of these bands appeared to be equal between the two clones and therefore may have resulted in the incomplete separation of S and R aphids by masking the proportion of activity contributed by E0.34 (Figure 3.4). However, in other PAGE gels, these additional bands were not always seen, suggesting their activity would be too low to be of significance in the esterase assay.

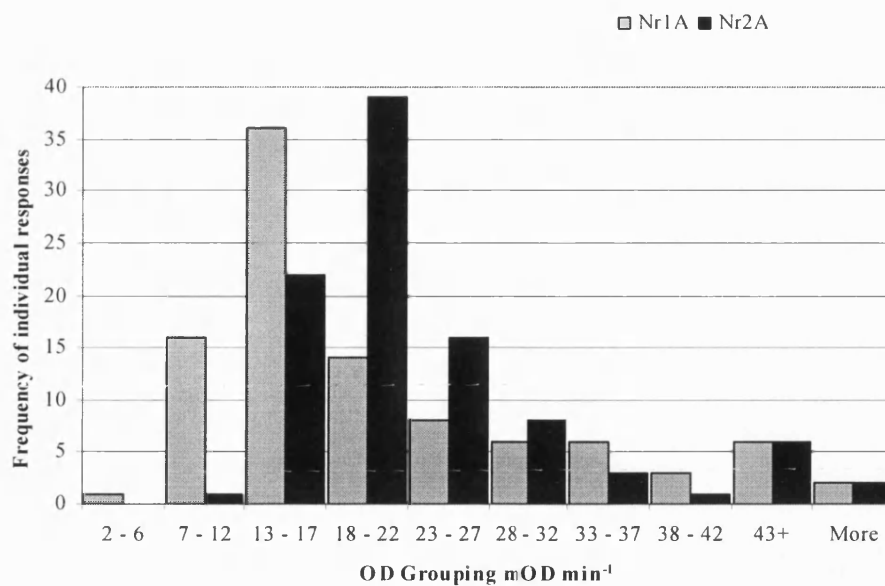


Figure 3.3 Optical density frequencies in a total esterase assay of 48 individuals of Nr1A (grey bars) and 48 individuals of Nr2A (black bars). The overlap in response between the two clones prevented the total esterase assay from being used as a rapid pirimicarb resistance diagnostic without further modification.

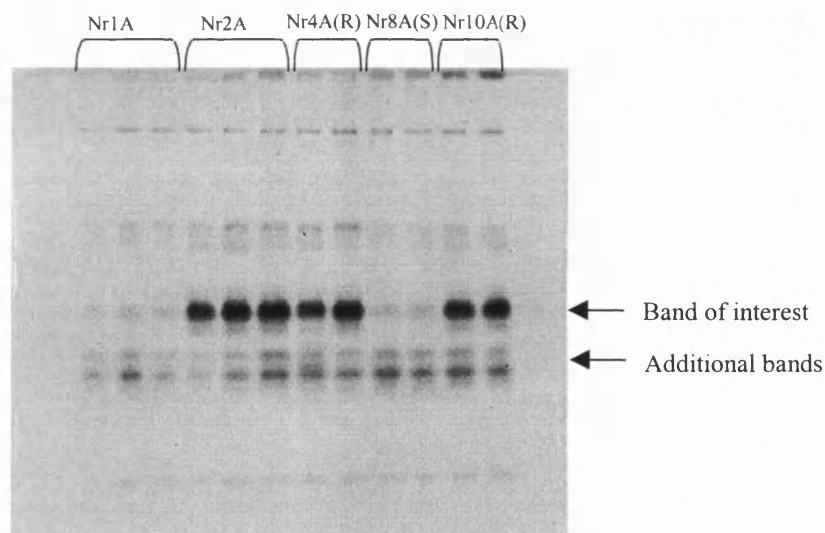


Figure 3.4 Esterase banding patterns in five clones of *N. ribisnigri* showing both the esterase associated with resistance and additional bands with a stain intensity which is equal between strains (S, susceptible R, resistant).

3.3.1.1. Effect of pH

Increasing the pH did not increase the esterase activity rates and at pH 9 the activity decreased (Figure 3.5). A combination of pH6 and 7 buffers was therefore used for all subsequent esterase related experiments.

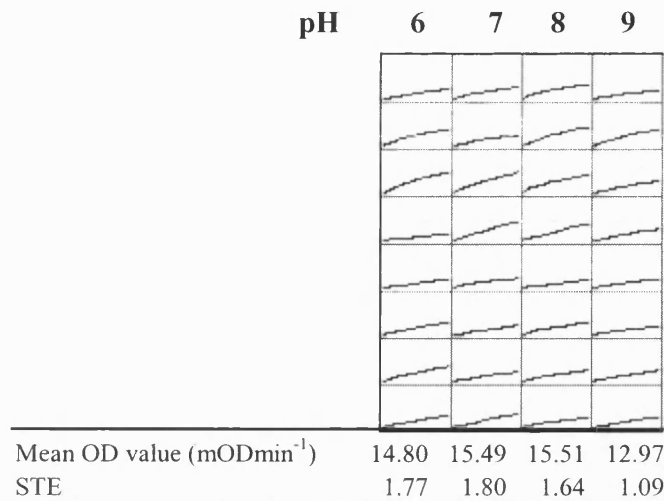


Figure 3.5 The effect of pH of the homogenisation and substrate buffers on total esterase activity in single Nr2A aphids. STE = standard error.

3.3.1.2. Effect of homogenate concentration

The amount of aphid homogenate in the total esterase assay directly influenced the total esterase value, increasing with an increase in proportion of homogenate present (Figure 3.6).

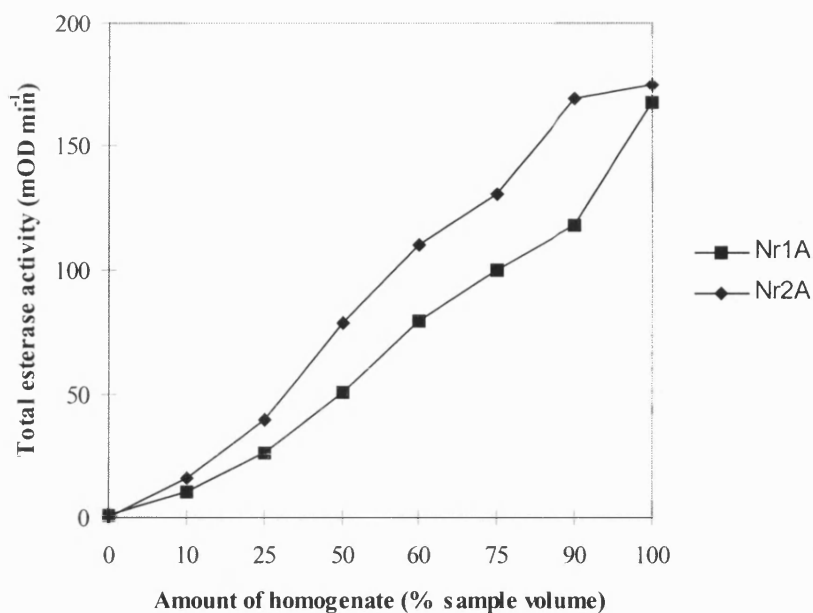


Figure 3.6 Effect of aphid homogenate concentration on total esterase activity. Percent sample volume represents the dilution of supernatant before the addition of assay reagents.

3.3.1.3. Total protein concentration

There was no significant correlation between esterase activity and protein concentration (linear regression: $F_{1,174} = 1.8$, $P = 0.18$). The majority of Nr1A and Nr2A individuals exhibited an esterase activity of between 10 and 20 mOD min⁻¹ and a protein content ranging from 15 to 30mg/ml (Figure 3.7). When individual esterase values were plotted after accounting for protein content, the frequency of response still overlapped (Figure 3.8).

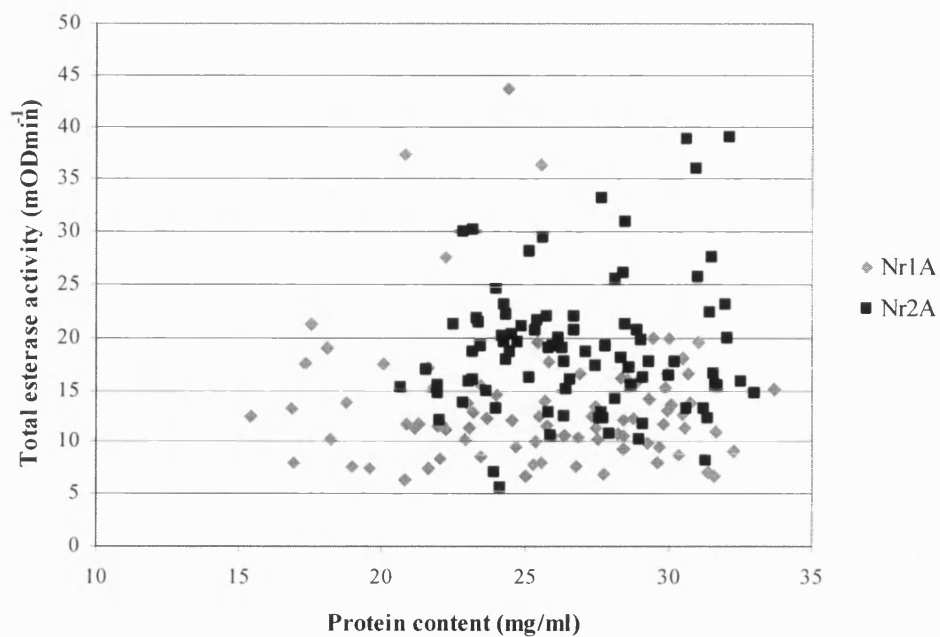


Figure 3.7 Protein content and esterase activity of single aphids of Nr1A and Nr2A. Independence of esterase activity from amount of protein prevented S and R being clearly distinguished by this method.

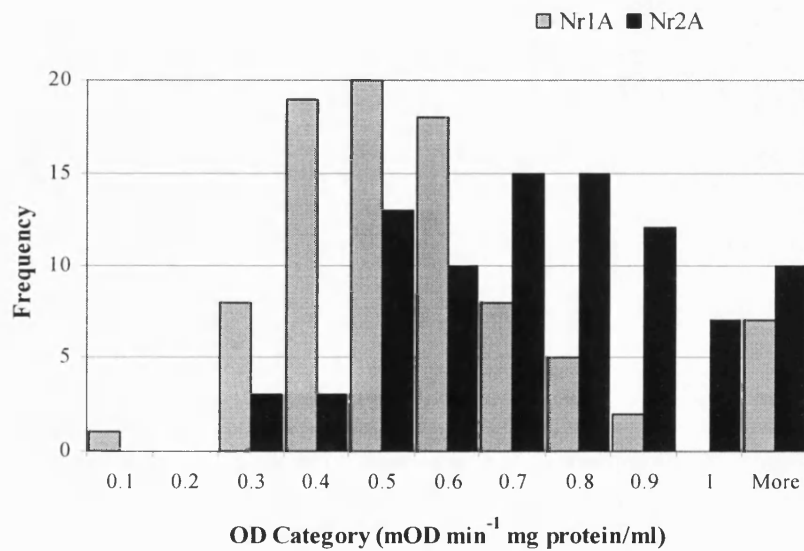


Figure 3.8 Esterase activity per mg/ml of protein. When protein content was accounted for the same overlap in response was seen between S and R.

3.3.1.4. Assay run time

Initial experiments extending the assay time with eleven individuals of Nr1A and Nr2A showed complete separation of responses after 30minutes, the distinction becoming even clearer after 90minutes (Figure 3.9). When this method was extended to 48 individuals from both clones, the separation was less clear although the homogeneity of response with clones improved with time (Figure 3.10). The response of Nr2A did not increase over time but the response of Nr1A decreased and became more uniform within the clone. After 90minutes, the distinction became less clear again as the values of Nr2A also began to decrease and therefore overlapped with the Nr1A response. When the extended assay was used to investigate other populations of *N. ribisnigri* available at the time, it could be seen that in the early stages of the assay, values were uniformly high. Over time, the mean esterase activity decreased but at different rates for each population, resulting in a greater distinction between activity values after 90 minutes (Figure 3.11). After 120 minutes the activity levels plateaued and no further change was seen. The values at 90 minutes however, still appeared to be independent from the E0.34 activities seen on PAGE gels. For example, the values of Nr1A and Nr2A were very similar and would not be distinguished if the method was used as a pirimicarb resistance-screening assay.

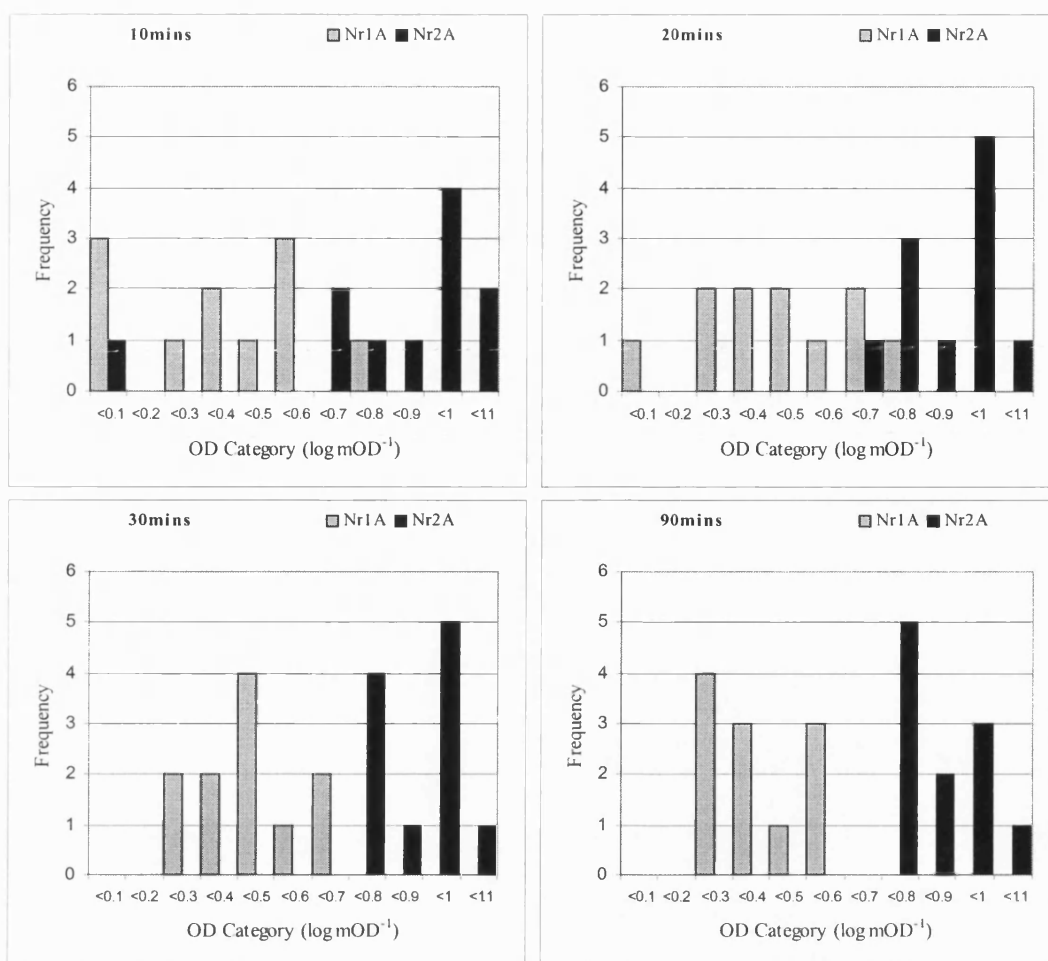


Figure 3.9 Frequency of esterase activity by eleven individuals of Nr1A and Nr2A over the course of an extended total esterase assay. A longer run time increased the separation of S and R individuals.

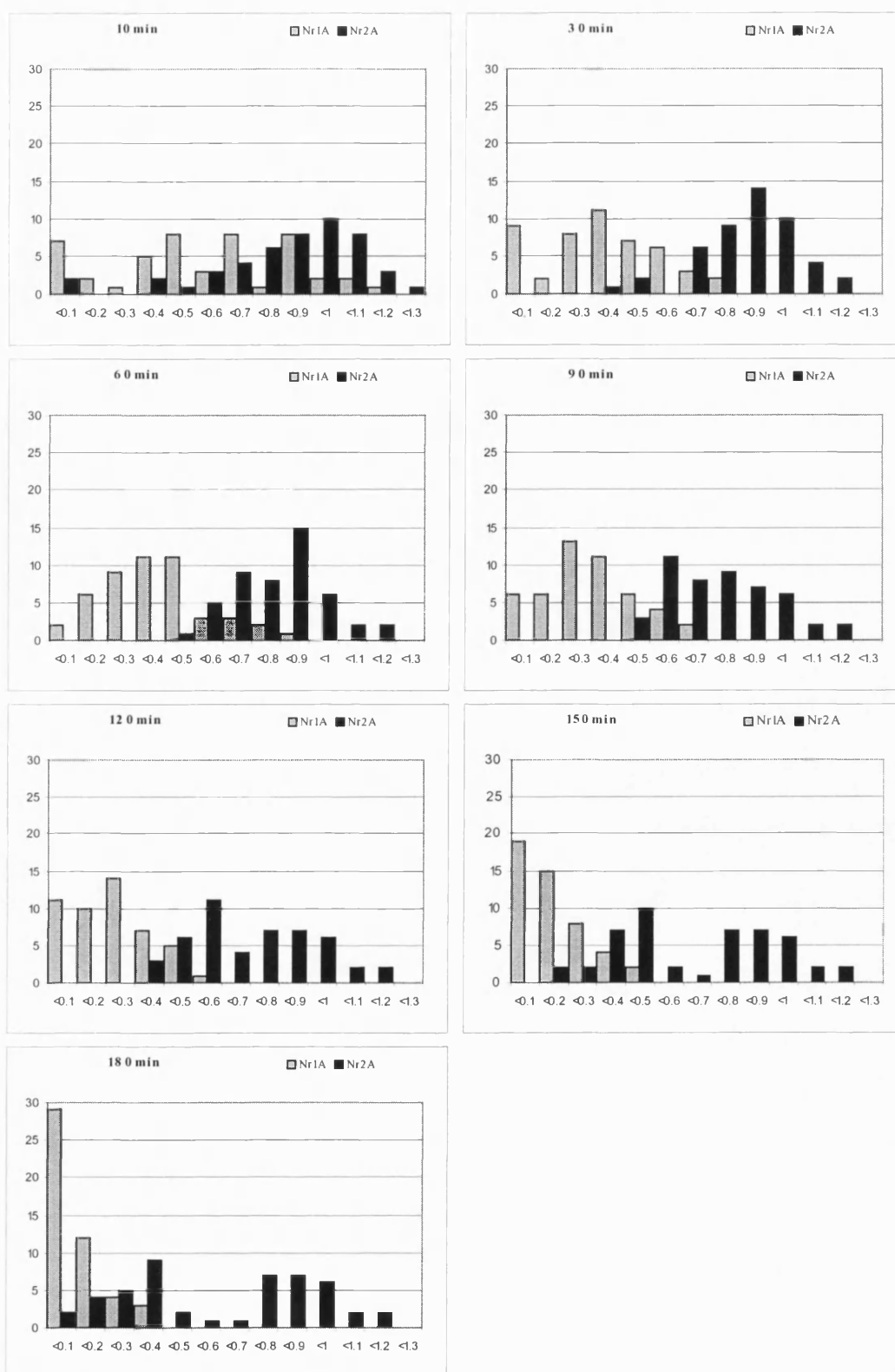


Figure 3.10 Frequency of esterase activity by 48 individuals of Nr1A and Nr2A over the course of an extended total esterase assay. x-axis = OD Category (log mOD⁻¹). y-axis = Frequency. By testing more individuals the distinction between S and R was made clearer after 90minutes but was not fully diagnostic of pirimicarb resistance.

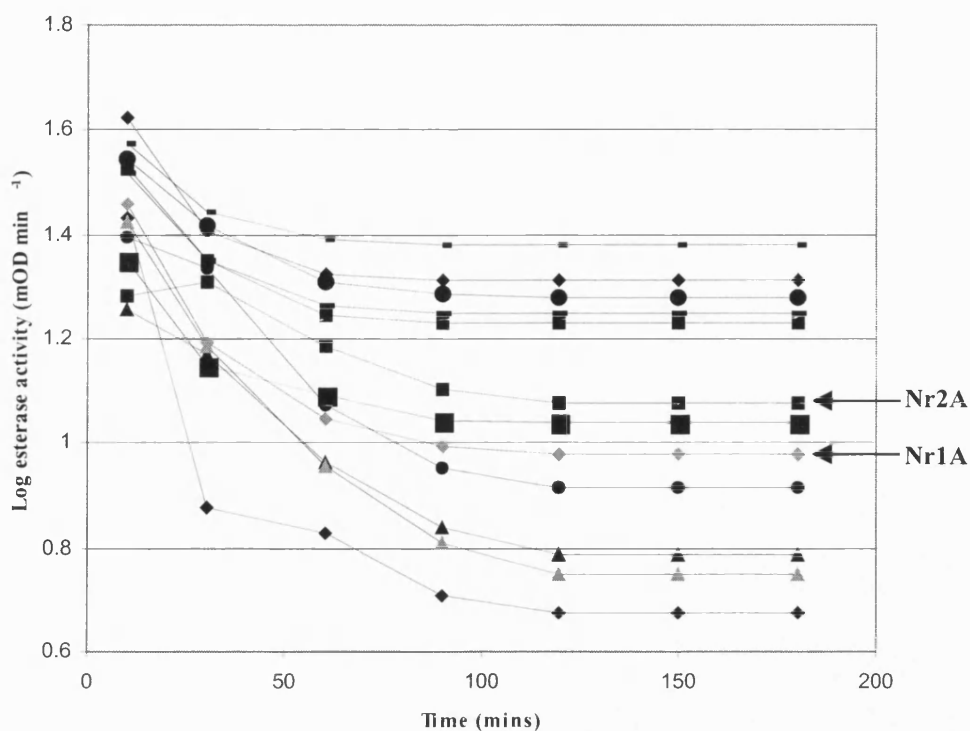


Figure 3.11 Mean esterase activity values of six individuals from twelve different populations plotted over time. While a longer assay time spreads out the response values, the final value did not allow a good assessment of E0.34 quantity to be made. For example, the value for Nr1A after 90 minutes is still very close to that of Nr2A.

3.3.1.5. Role of AChE

The addition of eserine to homogenates of Nr1A and Nr2A caused the AChE activity to be reduced 46-fold and 43-fold respectively. However, total esterase activities were not significantly altered (Figure 3.12).

	Esterase		AChE	
	Nr1A	Nr2A	Nr1A	Nr2A
No Eserine	41.058	104.03	653.10	878.10
10 ⁻⁶ M Eserine	36.819	90.616	14.105	20.602

Figure 3.12 Comparison of esterase and AChE activity with and without addition of eserine to the aphid homogenate. Data taken directly from Softmax output and measured in mOD min⁻¹.

3.3.2. Esterase stability

PAGE analysis of an Nr2A homogenate over time showed that there was a gradual reduction in activity of E0.34. However, while there was a clear difference between 0min and 455mins, there was little change in stain intensity after this point. A simultaneous total esterase assay correlated well with the PAGE result, showing a reduction in value over time, but there were two points at which a significant drop in activity occurred, one immediately after homogenisation (10mins) and another 135mins after homogenisation (Figure 3.13).

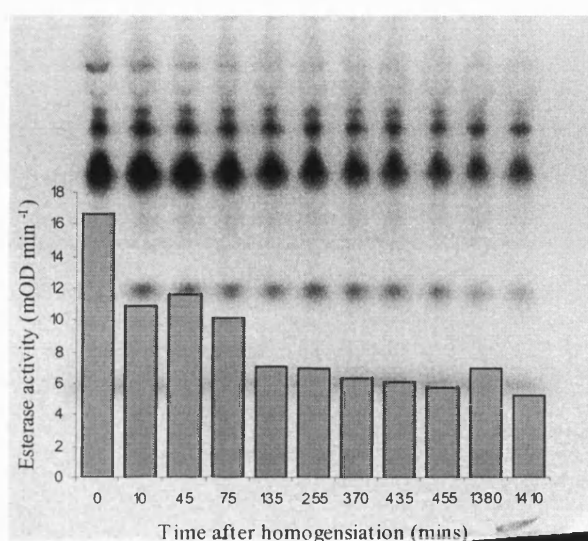


Figure 3.13 Monitoring the effect of time on esterase activity by both PAGE (banding pattern) and total esterase (bar chart). While the PAGE analysis suggested there is no sudden reduction of activity over time, total esterase analysis showed time was an important factor in assessing esterase activity.

3.3.3. Effect of pirimicarb on esterase activity

Figure 3.14 shows that the activity of E0.34 in an Nr2A homogenate (as visualised on a PAGE gel) was completely inhibited after incubation with pirimicarb. Total esterase analysis of the same homogenate showed activity to be reduced by only around 2-fold compared with around an 8-fold reduction in T1V (Table 3.1). Similarly there was a 1.7-fold decrease in total esterase activity in Nr1A and Nr4A (Table 3.2).

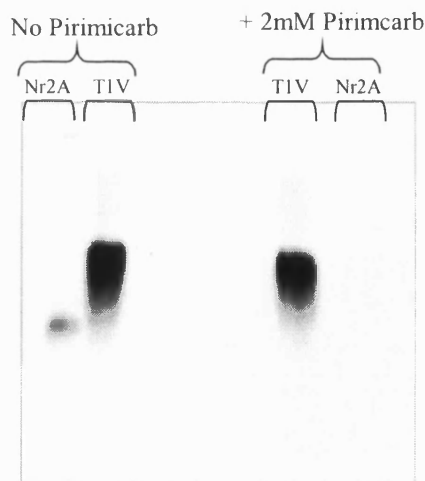


Figure 3.14 A PAGE gel showing E0.34 in *N. ribisnigri* (Nr2A) and E4 in *M. persicae* (T1V) with and without the addition of pirimicarb to the aphid homogenate.

Table 3.1 The effect of pirimicarb on total esterase activity in *N. ribisnigri* and *M. persicae* over time (mOD min⁻¹).

Pirimicarb Conc.	30min		90min		180min	
	0mM	2mM	0mM	2mM	0mM	2mM
Nr2A	39.4	22.8	44.4	22.0	55.0	22.4
T1V	253.5	65.3	192.0	26.4	244.2	24.8

Table 3.2 The effect of pirimicarb on total esterase activity in Nr1A and Nr4A after a 30min incubation (mOD min⁻¹).

Nr1A		Nr4A	
0mM Pir	3mM Pir	0mM Pir	3mM Pir
62.5	38.75	57.75	33.75

3.3.4. Radiolabelling

Radiolabel binding studies showed an increase in the amount of [³H]-DFP at the E0.34 position in Nr2A compared with Nr1A demonstrating that, as with *M. persicae* E4, the elevation in esterase activity is caused by overproduction of the esterase rather than the presence of a more active form (Figure 3.15).

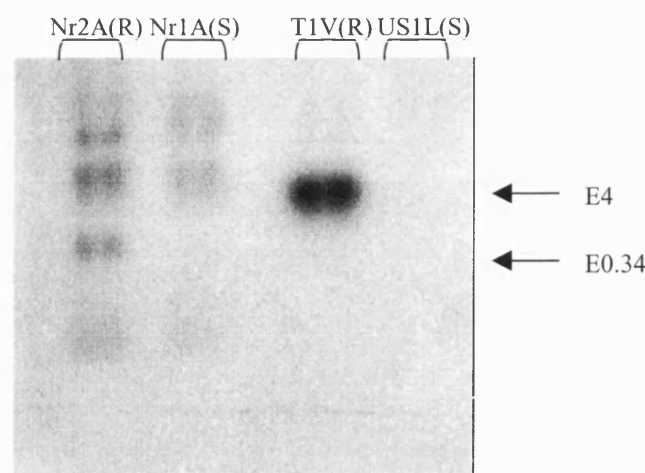


Figure 3.15 X-ray film of radiolabelled (DFP) esterases in susceptible (S) and resistant (R) clones of *N. ribisnigri* (Nr1A/Nr2A) and *M. persicae* (US1L/ T1V). Darker staining in the position of the resistance associated esterases in both species implicates the overproduction of esterases as the cause of elevated esterase activity.

3.4. Discussion

The ten minute total esterase assay failed to discriminate between pirimicarb resistant and susceptible individuals on the basis of esterase activity. In the PAGE analysis described in chapter 2, the difference in activity of E0.34 between S and R was very apparent, but by assessing total esterase activity, a similar discrimination was not seen. While results showed the average esterase activity of Nr2A (R) was greater than Nr1A (S) when the mean of several readings was taken, the range of results obtained from individuals of a single clone resulted in an overlap of response when comparing S and R individuals. Therefore, in order to improve the resolution of the assay, variables that may have influenced E0.34 activity and the separation of the clones were investigated.

It was found that the esterase activity measured by the total esterase assay was stable within the pH range 6-9 and the use of variable homogenate concentrations confirmed that the assay was correlated to the amount of homogenate present in the assay. In addition, in all experiments that used a mass homogenate, the esterase activity of Nr2A was always greater than that of Nr1A. The exception to this was the point at which 100% homogenate was used when values of Nr1A and 2A were

almost the same. It is possible that the homogenised pellet was disturbed in the Nr1A sample and entered the supernatant fraction thus causing a higher esterase reading.

In addition to pH and homogenate concentration, it was important to establish that the difference in esterase activity between Nr1A and 2A was not a result of Nr2A individuals being larger. The results showed that esterase activity was independent of protein content and when total esterase values were adjusted for protein, the overlap of responses still occurred.

From the results of the ten minute assays, it was seen that poor discrimination between S and R clones resulted from initial inconsistent responses of individuals within a clone. Increasing the run time provided the first improvement in S and R distinction. The distinction occurred as a result of responses within a clone becoming more uniform rather than as a shift in activity. However, activity in Nr1A did decrease slightly over time. When larger samples were tested, the distinction was not complete but considerably clearer after 90min compared with 10min. After running the assay for 150mins, the activity of Nr2A also began to decrease which caused a similar overlap to that seen after 10minutes. The effect of extending the assay run time was clearly seen when it is applied to several different populations and the mean result plotted over time. While the assay began with many populations exhibiting a similar high esterase activity, this decreased until a plateau was reached after which point very little change occurred. However, while the results became more separated and uniform within a population, the difference between S and R was still not at all clear.

E0.34 activity, as visualised on a PAGE gel, was completely inhibited after a 30min incubation with pirimicarb. However, the total esterase assay showed that approximately half of the original esterase activity remains. This suggests that there was another enzyme reacting with the model substrate 1-naphthyl acetate that did not appear on PAGE gels. In addition, its activity was not inhibited by the presence of pirimicarb. This unidentified enzyme appeared to contribute approximately half of the esterase activity found in individual aphids and was therefore, potentially, the cause of the poor discrimination of E0.34 levels in the total esterase assay.

Additional evidence for this enzyme was the reduction of total esterase activity in single Nr2A at around 150 and 180 mins into the assay. When compared to a mass homogenate that was incubated at the same temperature, a drop in total esterase activity was recorded 135mins after homogenisation but this drop was not reflected by any change in E0.34 activity on the PAGE gel. This suggested that the unidentified enzyme was less stable than E0.34 at room temperature.

Although during the course of the project, additional esterase bands were seen on PAGE gels, both above and below E0.34, lack of reproducibility of these bands suggested that their activity would not be sufficient to contribute half of the total esterase activity of a single aphid. In addition, these esterases were also inhibited by pirimicarb. The MACE screening assays shown in 2.3.2.2 demonstrated that *N. ribisnigri* AChE is inhibited by pirimicarb. In addition, by using the known AChE inhibitor, eserine, any role of AChE in the hydrolysis of 1-naphthyl acetate was ruled out.

Radiolabelled DFP showed the elevation in esterase activity to be related to an over-production of E0.34 rather than the production of a modified esterase. While it is known that this phenomenon is caused by gene amplification in *M. persicae* (Field *et al*, 1997), it is possible that increased transcription or post translational modification, rather than gene amplification *per se*, could also be occurring in *N. ribisnigri*.

Investigations of E0.34 showed:

- Elevation of activity in E0.34 was caused by over-production of the esterase.
- The ten-minute total esterase assay did not distinguish between pirimicarb S and R individuals.
- Discrimination between S and R was improved by running the total esterase assay for longer but still it was difficult to categorise populations.
- E0.34 was still active after 23hrs at room temperature but activity was lost during this time.
- E0.34 activity was inhibited by pirimicarb but not by eserine.

- The esterases visualised on a PAGE gel may only represent 50% of the total esterase activity measured using the total esterase assay.
- Another enzyme, capable of hydrolysing 1-naphthyl acetate, which was unaffected by pirimicarb and was not seen on a PAGE gel may have been contributing to the remaining 50% of the total esterase activity.

4. PURIFICATION AND CHARACTERISATION OF ESTERASES

4.1. Introduction

Purification of the proteins which showed activity for the substrate 1-naphthyl acetate in *N. ribisnigri* would allow the interactions of E0.34 with pirimicarb to be studied in greater detail and also provide an opportunity to identify the unknown source of 50% of the aphid's esterase content. If E0.34 was shown to be responsible for pirimicarb resistance, purification of E0.34 would also provide an opportunity to develop an E0.34 specific immunoassay for rapid diagnosis of pirimicarb resistance.

4.2. Materials and Methods

4.2.1. Esterase purification

Three methods were used to purify E0.34.

4.2.1.1. Ion-exchange chromatography

Initial attempts to purify E0.34 followed the methods of Devonshire (1977) using gel filtration and ion-exchange chromatography. Nr2A aphids (0.5g) were homogenised in 5ml pH6 MES, centrifuged at 1500g for 5min and the supernatant taken. Low molecular weight material was removed using a PD10 column (Pharmacia Biotech) containing Sephadex G25. The homogenate was loaded onto a column (5cm x 1cm) of strong anion-exchanger, QAE - Sepharose fast flow (Pharmacia Biotech), and eluted with a linear 0-1M NaCl gradient in 100ml pH6 MES. 2ml fractions were collected and 20µl samples assayed for esterase activity over five minutes using the method described in 3.2.1. Fractions showing esterase activity were desalted and concentrated using a Miniplus concentrator (Millipore Corporation). 20µl of the concentrated samples were run on PAGE gels as described in 2.2.4.2 in order to locate the fractions containing E0.34.

Following the findings of isoelectric focusing (see 4.2.5), BuChE activity was also assessed in 20µl samples from all fractions using the method described in 4.2.7.

In addition, peaks showing high esterase activity were assessed for AChE activity using the method described in 2.2.4.3 without the addition of pirimicarb.

In order to determine the elution profile of E0.34 from a Q ion exchange column, 3ml of E0.34, previously purified by electro-elution (see below) was loaded onto a Q column and run as described above using a 1M NaCl elution gradient.

4.2.1.2. Electro-elution

Nr2A aphids (1g) were homogenised in 5ml pH7 PB and centrifuged at 1500g for 5min. The supernatant was taken and low molecular weight material was removed from the crude homogenate by passing it through a column (2.5cm x 25cm) of Sephadex G-25. The ~15ml sample recovered from the column was concentrated to a 4ml volume using a Miniplus concentrator. Sucrose (0.5% final volume) was added to the concentrate before loading the sample in equal volumes onto four PAGE gels and run as described in 2.2.4.2. Borders (1cm wide) were 'crinkle cut' from the gels, rinsed in pH 6.0 PB for 30min then stained in the same buffer containing 2.5mM Fast Blue RR, 1% acetone and 0.6mM 1-naphthyl acetate for 10min. The stained borders were realigned with the respective gels and the band of interest cut from the unstained sections. Slices were diced and electro-eluted in Tris-base/glacial acetic acid (0.6%/0.15%) at room temperature for 4h. The eluted esterase was recovered and stored at -20°C until required. Recovery was monitored by the assessment of protein content using the Bradford assay as described in 3.2.1.3 and the procedure repeated as necessary.

4.2.1.3. Preparative electrophoresis

An Ornstein Davis gel (see 2.2.4.2) was cast in a Bio-Rad Prep Cell preparative electrophoresis tank (1cm:3cm stacking:resolving gel). Nr2A aphids (0.05g) were homogenised in 3ml pH6 MES, loaded onto the gel and run in a discontinuous buffer system (Davis, 1964) for 6hrs at 12W constant power and 600V. Nine 25ml samples were eluted from the tank in pH6 MES after 5hrs at a rate of 1ml/min. To ensure E0.34 had been eluted from the end of the gel, the gel was removed and stained as described in 2.2.4.2. 25µl of each sample was run on a PAGE as described in 2.2.4.2. Those fractions showing E0.34 activity were concentrated using Miniplus concentrators.

4.2.2. Esterase reactivation studies

Recovery of E0.34 after incubation with pirimicarb was monitored over time using the 10 minute kinetic total esterase assay described in 3.2.1. To ensure the total esterase assay was sensitive to E0.34 concentration, a serial dilution of E0.34 was run in the assay before beginning the re-activation studies. Once confirmed, a sample of purified E0.34, with an uninhibited total esterase value of $>100 \text{ mODmin}^{-1}$ per assay, was incubated in 5ml pH 7.0 PB containing pirimicarb (10^{-3}M) and bovine serum albumin (0.25%), included to maintain enzyme stability, until esterase inhibition was $>90\%$. The 5ml sample was loaded onto a Sephadex G-25 (2.5 X 25cm) column. Previous studies using radiolabelled DFP had established that the insecticide-bound esterase came off the column in the 40-45ml fraction, 10ml before the unbound insecticide was released. Sub-samples (80 μl) of the 40-45ml fraction were screened for esterase activity over a 6h time course. Because of background hydrolysis in the absence of esterase activity, fresh solutions were made at intervals throughout the experiment.

4.2.3. Effect of detergent concentration

The optimum detergent concentration for E0.34 activity was established using a serial dilution of Triton X-100 in the total esterase assay. A serial dilution of Triton X-100 was made with pH7 PB in a microplate of 0.5%, 0.17%, 0.06%, 0.02%, 0.006% and 0% of the final assay volume. 10 μl purified E0.34 was added to each well and the reaction initiated by the addition of 100 μl of substrate as described in 3.2.1. In addition, two PAGE gels were cast as described in 2.2.4.2 with and without 0.2% Triton X-100. Both gels were run with one 20 μl sample of pure E0.34, derived by electro-elution, and 20 μl of 3 Nr2A (homogenised in 45 μl pH7 PB containing 5% sucrose/1.6%Triton X-100). Both gels were stained as described in 2.2.4.2.

4.2.4. Enzyme linked immunosorbent assay (ELISA)

C. 0.5mg of purified E0.34 was conjugated to keyhole limpet haemocyanin using gluteraldehyde and emulsified in Freund's complete adjuvant before being injected intramuscularly into a rabbit. One month later a further 0.5mg of sample was injected following which five blood samples were collected at 2 week intervals. Serum was separated by centrifugation and stored at -20°C . A pre-bleed was taken before injecting the esterase, to act as a control.

Forty Nr2A individuals were homogenised in 3ml coating buffer (0.2M sodium carbonate, pH9.6), centrifuged at 1500g for 5min and the supernatant taken. 50µl of supernatant was added to 50µl of the same buffer in 48 wells of a 96 well microplate, mixed and incubated at 37°C for 1hr. The plate was washed 3 times with pH7.4 PBS buffer containing 0.05% Tween 20 (PBS-Tween). Wells were blocked with 200µl extraction buffer (PBS-Tween 20 buffer containing 0.5% NIDO milk powder) and incubated at 25°C overnight before washing as before. 2µl of each bleed was diluted in 1ml extraction buffer and 150µl added to the first row of the microplate. 100µl extraction buffer was added to all other wells and 50µl of serum transferred down the plate to create a serial dilution for all six bleeds. No serum was transferred to the final row. After a 1hr incubation at 37°C, the plate was washed as before. 5µl anti-rabbit IgG (Sigma) was diluted in 10ml extraction buffer. 100µl was added to all wells and incubated at 37°C for 1hr before washing as before. To each well, 100µl 1mM 1-naphthyl acetate was added and incubated for 15min. 50µl 4mM fast blue BB salt was added and an endpoint reading taken at a wavelength of 450nm using a Thermomax microplate reader (Molecular Devices).

Following this preliminary examination, one bleed showing the greatest binding affinity was selected and tested for binding specificity to E0.34 using a serial dilution of aphid homogenate. 30 Nr1A or Nr2A individuals were homogenised in 190µl coating buffer, centrifuged and 150µl of supernatant put in one well of a 96-well microplate. To seven remaining wells of the column, 100µl coating buffer was added and 50µl of homogenate was transferred down the plate creating a dilution series. One well was left with no homogenate to act as a control. The ELISA was conducted as described above using 4µl of serum diluted in 2ml extraction buffer and 100µl added to all wells.

4.2.5. Isoelectric focusing

Isoelectric focusing was conducted using 1mm thick pH3.5-8.5 polyacrylamide ampholine PAGplates (Amersham Pharmacia Biotech) with 1M phosphoric acid as the anode solution and 1M sodium hydroxide as the cathode solution. 20µl of sample was applied to individual application pieces placed approximately at the

position of pI 7.0 and the gel plate run at 4°C, 400V for 2.5hrs. Gels were stained for esterase activity overnight in 0.2M phosphate buffer, pH6 containing 2.5mM Fast Blue RR, 1% acetone and 0.6mM 1-naphthyl acetate. Gels were fixed and stored in 7% acetic acid. To determine the pI values of the esterases, 20µl of broad range (pH 3-10) calibration solution (Amersham Pharmacia Biotech) was run alongside the samples of interest. After running, the section of the gel plate containing the markers was cut and stained for protein with Coomassie brilliant blue. The remaining gel was stained for esterase activity as described above and the pI of the esterases estimated by aligning with the calibration gel strip.

During optimisation of the process, some of the PAGplates used were soaked in MES6 buffer containing 1% Triton X-100 for 1hr prior to running and stained for esterase activity in the presence of 0.1% Triton X-100.

4.2.6. Butyrylcholinesterase activity staining

A PAGE gel was run as described in 2.2.4.2 containing a sample of Nr1A and US1L aphids. Staining for BuChE activity followed the method of Karnovsky and Roots (1964). The gel was washed for 15min in water and stained for 1hr in water containing 3mM CuSO₄, 17mM glycine, 0.1mM sodium acetate and 9mM butyrylthiocholine iodide (BTChI). The gel was then washed with water and stored.

4.2.7. Butyrylcholinesterase assays

Assays characterising the levels of BuChE in Nr1A and Nr2A populations were based on the AChE assay described in 2.2.4.3. Single aphids (8 per clone) were homogenised in 50µl pH7 PB in separate wells of a 96-well microplate and 50µl DTNB added. Assays were started by the addition of BTChI in buffer (100µl) to give a final substrate concentration of 0.5mM and a final DTNB concentration of 15µM. Assays were monitored for 20 min using a Thermomax microplate reader (Molecular Devices), utilising SOFTmax software that subsequently fitted linear regressions to successive absorbance readings taken at a wavelength of 405nm from each well. The experiment was repeated using pH9 Tris base 0.02M buffer containing 0.1% Triton X-100

4.2.7.1. Effect of pirimicarb on BuChE activity

Two rows of a 96 well microplate were filled with 30µl pH9 Tris base 0.02M containing 0.1% Triton X-100 (pH9 TB). 3µl 10⁻¹M pirimicarb was added to the first well of each row and 10µl transferred through the row to create a serial dilution. No transfer was made to the last wells of each row. 7µl pH9 TB was added to the two first wells in order to make all volumes 30µl. Twelve aphids of Nr1A or Nr2A were homogenised in 250µl pH9 TB, centrifuged at 1100g for 10s and 20µl added to each row. Homogenates were incubated for 10minutes and then assessed for BuChE activity by the addition of BTChI and DTNB as described in 4.2.7.

4.3. Results

4.3.1. Esterase purification

Figure 4.1 shows the esterase activity in fractions of Nr2A homogenate after elution from an anion exchange column. Two fractions were found to contain high esterase activity. From PAGE analysis, it was expected that E0.34 would exhibit an anionic surface charge and would therefore be contained in the fraction eluted during the salt gradient. However, an additional peak of activity was identified in a fraction collected before the salt gradient was begun. Repeating the experiment with smaller quantities of aphid homogenate showed that this was not a result of the column being overloaded. Samples of interest were desalted, concentrated to 1ml and run on PAGE.

E0.34 was found within a sample representing fractions 21 to 30 (between *c.* 0.2 and 0.3M NaCl) but its activity appeared low when compared to the pre-ion-exchange (post G-25) sample. When trying to separate the esterase bands further, E0.34 appeared to be very similar in ionic charge to the other esterases, thus making it difficult to purify. In addition, at each stage, E0.34 activity appeared to be reduced (Figure 4.2a and b). Comparison with the activity in the post-G25 product suggests that the process of ion-exchange was resulting in a very poor yield of E0.34. Fractions 0-10 (i.e. before the salt gradient) showed no esterase activity on PAGE gels.

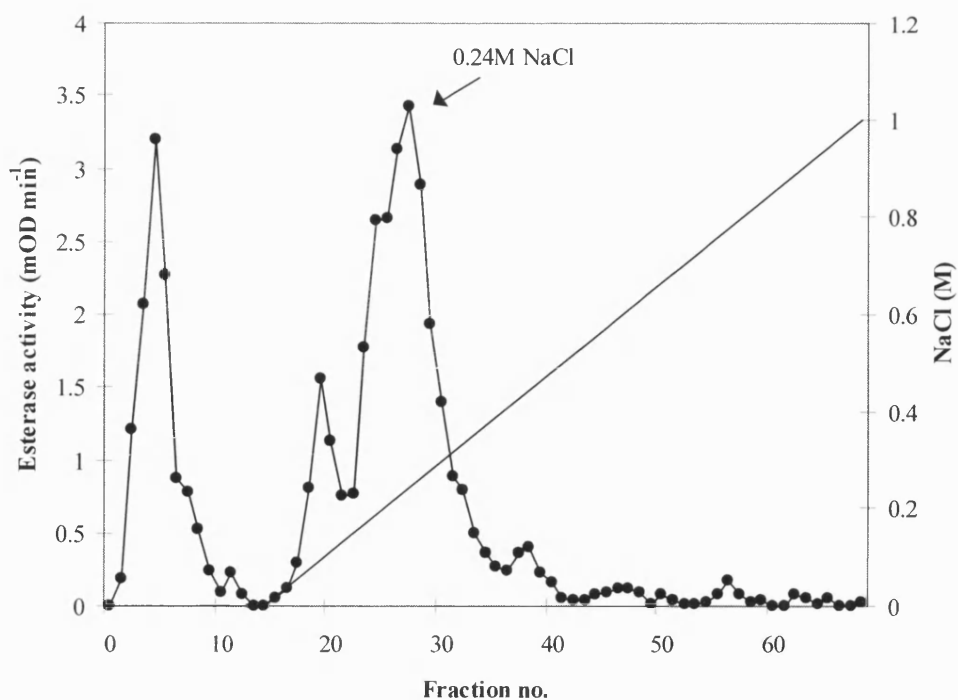


Figure 4.1 Esterase content of eluted fractions after passing an Nr2A homogenate through an anion-exchange column. Proteins which passed straight through the column were considered to have a cationic surface charge. Proteins with an anionic surface charge bound to the column and were eluted using a 0-1M NaCl gradient (straight line).

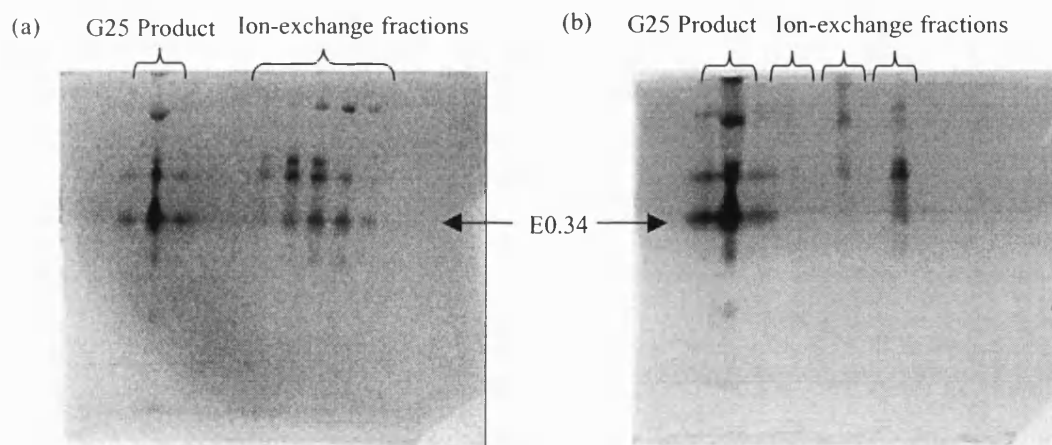


Figure 4.2 After locating most of the esterase activity in fractions 21-30 (see Figure 4.1), they were pooled and run through another anion exchange column using a more shallow NaCl gradient in order to separate the esterases further. (a) The results showed E0.34 to be very similar to the other esterases in terms of surface charge and that despite the concentrating process, compared to the post-G25 control, the E0.34 post ion-exchange appeared to be losing activity. (b) After another ion-exchange run the E0.34 has almost completely disappeared compared to the control.

Electro-elution allowed successful separation of E0.34 from other esterases (Figure 4.3). However, the process was more resource consuming than column purification, requiring approximately 20g of Nr2A to yield c. 1mg of purified protein.

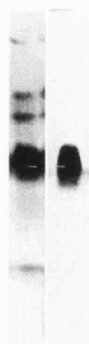


Figure 4.3 Comparison of banding patterns of crude Nr2A homogenates (left gel strip) and purified E0.34 after electro-elution (right gel strip).

Preparative electrophoresis also provided a method by which E0.34 could be separated from other esterases. Figure 4.4 shows three 25ml samples, recovered over a 1h 30min period, which showed E0.34 activity after concentrating and running on a PAGE gel. When pooled together, fractions 5, 6 and 7 provided a 2.5ml concentrated sample of E0.34.

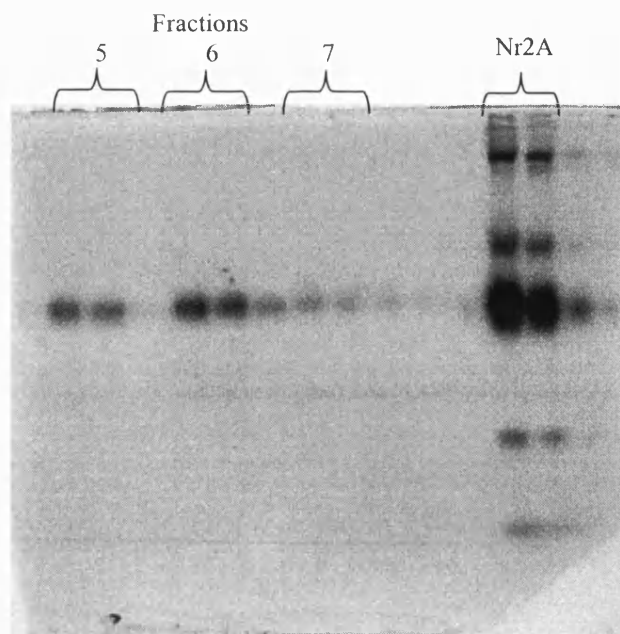


Figure 4.4 Three samples of elution product from preparative electrophoresis on a PAGE gel after being stained for esterase activity. A crude homogenate of Nr2A was run alongside the samples in order to indicate the position of E0.34. Each sample was run in two adjoining wells.

Both electro-elution and preparative electrophoresis provided samples of E0.34 which did not contain other esterases. Further studies could therefore be conducted in order to investigate specific properties of this enzyme.

4.3.2. Reactivation studies

A serial dilution of purified E0.34 in the total esterase assay demonstrated that, despite the poor discrimination between S and R aphids reported in chapter 3, the assay did register E0.34 activity (Table 4.1). The reactivation of E0.34 after incubation with pirimicarb could therefore be monitored using this assay.

After removal of excess inhibitor, recovery of E0.34 activity was minimal, recovering less than 4% activity over six hours, suggesting the major role of E0.34 in pirimicarb detoxification was by sequestration rather than rapid hydrolysis of the insecticide (Figure 4.5).

Table 4.1 Effect of E0.34 concentration on output from total esterase assay.

E0.34 concentration (μg of protein)	Esterase activity (mOD min^{-1})
0.015	5.2
0.007	1.7
0.004	0
0	0

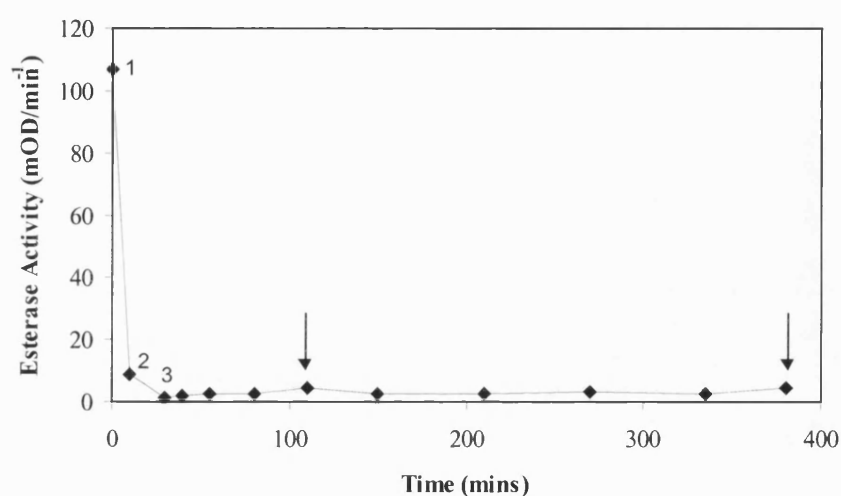


Figure 4.5 Following the addition of pirimicarb to a sample of pure E0.34, excess inhibitor was removed and recovery of esterase activity was monitored over time. Point 1 = Before addition of pirimicarb. Point 2 = After 10min incubation with pirimicarb. Point 3 = After removal of excess pirimicarb. Arrows indicate where the substrate for the total esterase assay was renewed, resulting in a slightly higher value.

4.3.3. Effect of Triton X-100

When incubated with an increasing concentration of Triton X-100, the activity of E0.34 towards the substrate 1-naphthyl acetate was seen to increase (Figure 4.6). The point of greatest increase (around 0.01% Triton X-100 final volume) indicated the critical micelle concentration (CMC), below which the detergent has little or no effect on enzyme activity. In addition, when two PAGE gels, one with and one without the incorporation of Triton X-100 into the gel matrix, were compared after running a pure sample of E0.34 and a whole aphid homogenate, there was clearly greater E0.34 activity in the presence of Triton X-100 (Figure 4.7).

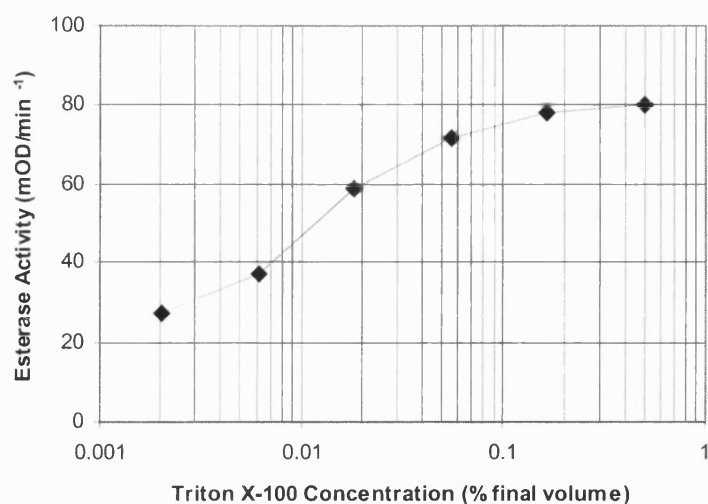


Figure 4.6 Incubation of the purified esterase, E0.34, with an increasing concentration of the detergent, Triton X-100, demonstrates an increasing turnover of the substrate, 1-naphthyl acetate.

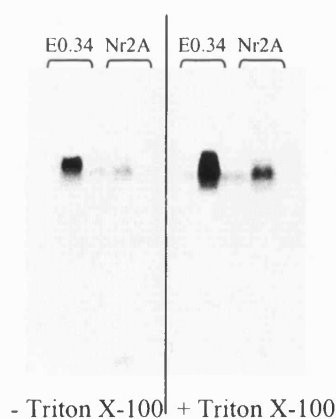


Figure 4.7 Two PAGE gels with (right) and without (left) 0.2% Triton X-100 incorporated into the gel matrix. 20 μ l purified E0.34 and 20 μ l of Nr2A mass homogenate were run on each gel and stained with 1-naphthyl acetate.

4.3.4. ELISA

Partial purification of E0.34 allowed polyclonal antibodies to be raised to the protein, potentially allowing a rapid diagnostic immunoassay for E0.34 to be created. Figure 4.8 shows that there was little difference in esterase binding properties between bleeds 2-5. However, whilst the serum was binding to a protein which exhibited esterase activity, it was not E0.34 because Nr1A values were greater than Nr2A (Figure 4.9).

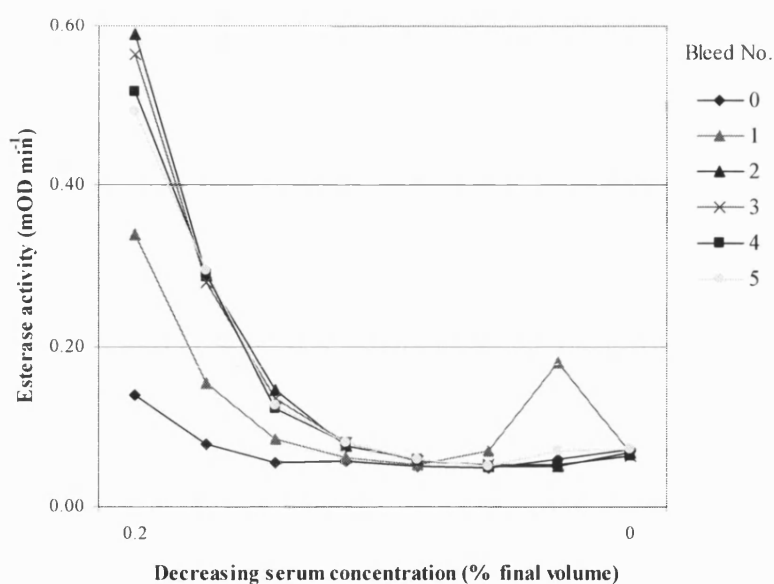


Figure 4.8 Binding properties of sera taken at different times after being exposed to purified E0.34.

Using serial dilutions of each serum in the presence of a standard concentration of aphid homogenate indicated the bleed with the greatest binding capacity. A pre bleed (0) was used as a control. Bleeds were taken at two week intervals after bleed 0.

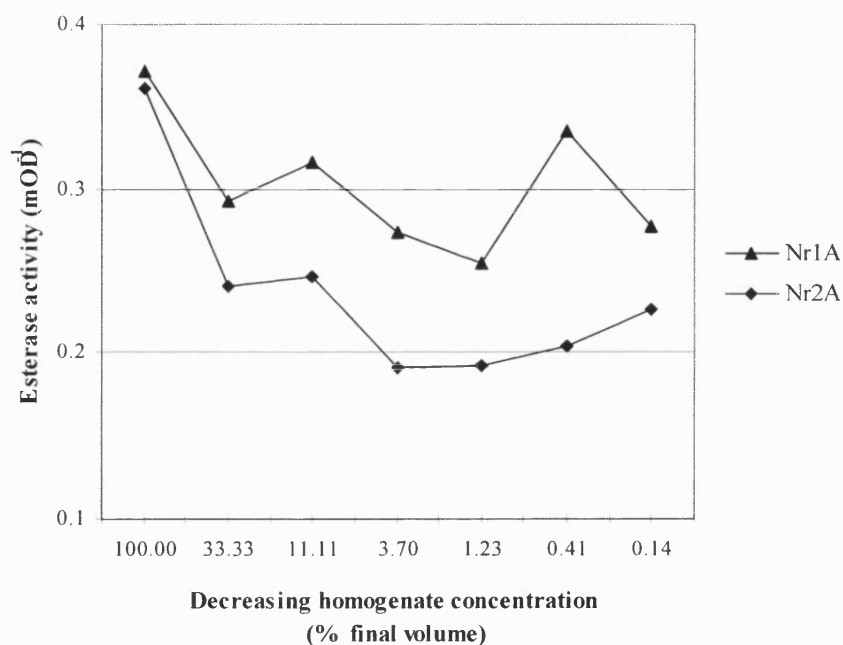


Figure 4.9 Bleed number 2 (see Figure 4.8) was used with a serial dilution of aphid homogenate to demonstrate if any binding capabilities were specific to E0.34. The result of the control, containing no homogenate, was subtracted from all values.

4.3.5. E0.34 and ion-exchange chromatography

When purified E0.34, prepared by either electro-elution or preparative electrophoresis, was passed through an anion exchange column the elution profile shown in Figure 4.10 was seen. As expected, the esterase was removed from the anion exchange column by the salt gradient. However, the esterase elution occurred at 0.6M NaCl compared with 0.24M NaCl when using a crude homogenate sample (see 4.2.1.1). This suggested that there was a difference in surface charge between E0.34 in a crude homogenate and when purified.

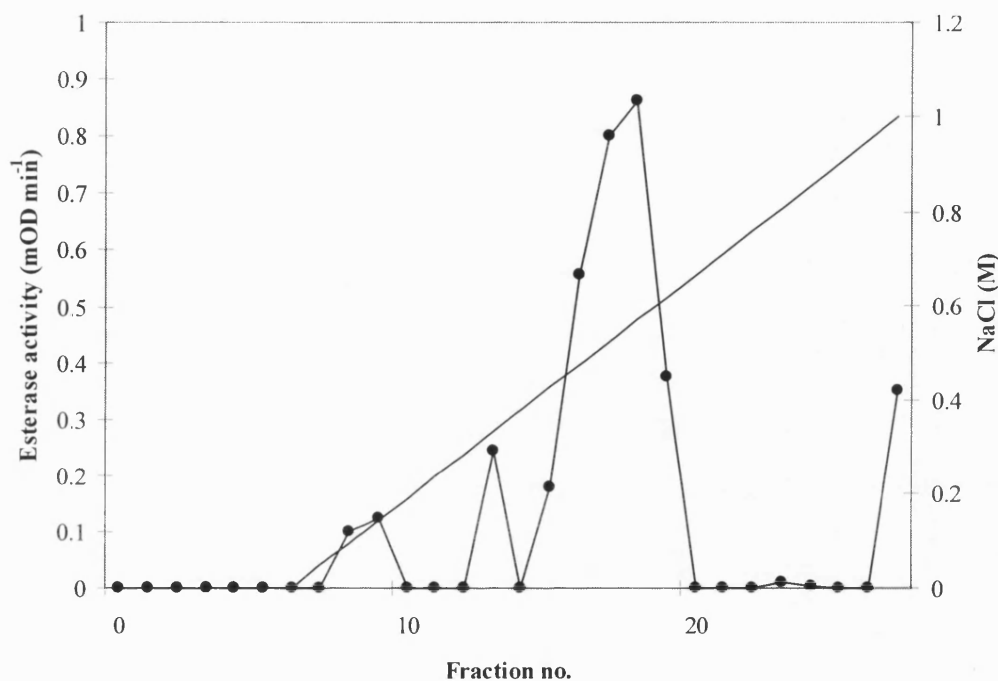


Figure 4.10 Elution profile after a purified sample of E0.34 (by electro-elution) has been passed through an anion-exchange column (QAE – Sepharose fast flow). Fractions were measured for total esterase activity. The profile suggests there are still some minor esterases present in the sample. The fraction containing E0.34 was eluted at around 0.6M NaCl.

4.3.6. Isoelectric focusing

The result of the ion-exchange purification suggested both anionic and cationic esterases were present in *N. ribisnigri*. In order to visualise both esterases an isoelectric-focusing (IEF) gel was run and stained for esterase activity. Samples of S (US1L) and R (T1V) *M. persicae* were included for a comparison.

The esterase bands seen varied depending on treatment of the IEF gel with Triton X-100. When the gel was soaked in a solution of Triton X-100 before and after running the gel, anionic esterase bands could be seen in both species, probably representing the same esterases as those seen on PAGE gels (Figure 4.11a). In the absence of Triton X-100, a cationic esterase band was seen in both *N. ribisnigri* clones (Figure 4.11b). In order to visualise both anionic and cationic bands on the same gel, the gel was pre-treated only with Triton X-100 solution. Anionic and cationic esterase bands could be seen in both *N. ribisnigri* clones but only anionic bands in *M. persicae* (Figure 4.11c).

Under these same conditions, the high esterase fractions pre NaCl gradient (cationic surface charge) and post gradient (anionic surface charge/E0.34) from the anion exchange column were run and stained for esterase activity. As expected, fraction 1 was seen to contain a cationic esterase (pI ~ 5.00) while fraction 2 migrated to the position expected for E0.34 (based on its relative mobility on PAGE gels) (pI ~ 8.00) (Figure 4.12).

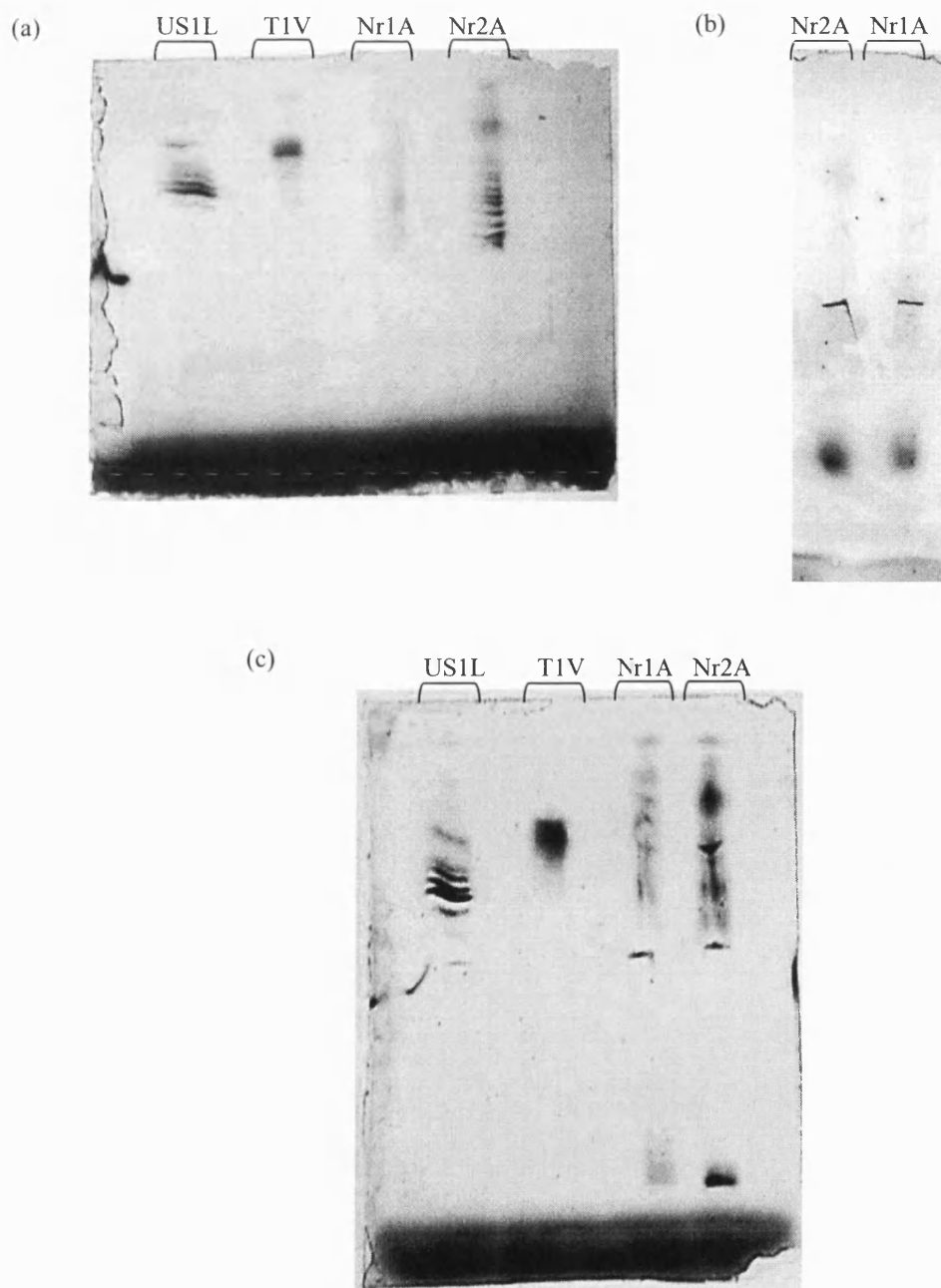


Figure 4.11 Isoelectric focusing gels stained for esterase activity. Samples were loaded in the middle of the gels. The top half of each gel represents the anionic region (i.e. attracts proteins with an overall anionic charge) while the bottom portion of the gel is the cationic region. (a) Gel pre-soaked and stained in 1% and 0.1% Triton X-100 solution respectively. (b) Gel run and stained in the absence of Triton X-100. (c) Gel pre-soaked in 1% Triton X-100.

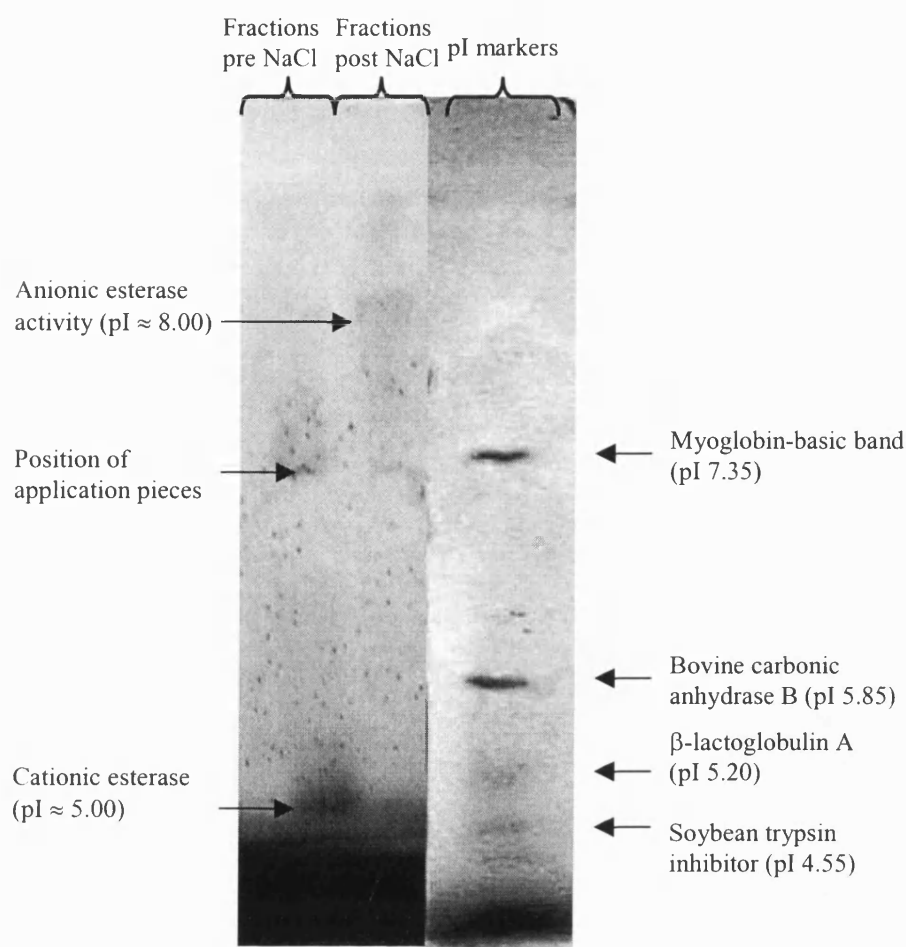


Figure 4.12 IEF gel showing separation of the two main esterase components of *N. ribisnigri* by anion exchange chromatography. By comparison with pI markers run at the same time, the approximate pI values of the two esterases were determined.

The sensitivity of the cationic esterase to the concentration of Triton X-100 (as shown in Figure 4.11) suggested that it might have been AChE, for which a critical detergent concentration has been reported in other insect species (Brestkin *et al.*, 1985). However, when compared with a mass homogenate (post G25), there was no significant activity against the substrate ATChI in either high esterase fractions (Table 4.2).

Table 4.2 AChE activity in a crude homogenate of Nr2A (post G25 purification) and in the two fractions showing esterase activity after separation by anion exchange chromatography (pre and post NaCl gradient).

	AChE activity (mOD min ⁻¹)
Post G25	49.112
Pre NaCl	1.037
Post NaCl	0.229

4.3.7. BuChE activity

When a PAGE gel was stained for BuChE activity, a white band could be seen in both *M. persicae* and *N. ribisnigri*. However, in *N. ribisnigri*, the band was at the most cationic portion of the gel (the top) suggesting that it was BuChE activity which was present in the cationic fraction after anionic ion-exchange (Figure 4.13). In addition, when the fractions collected by anion-exchange chromatography (see Figure 4.1) were tested for BuChE activity it could be seen that the cationic fraction (pre-NaCl) had high activity against the substrate BTChI (Figure 4.14). If both BuChE and general esterases were present, they appeared to react with both the substrates, as the peaks of troughs of activity against BTChI and 1-naphthyl acetate are mirrored in each fraction (Figure 4.14).

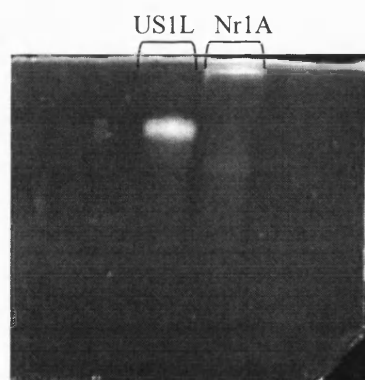


Figure 4.13 PAGE gel stained for BuChE activity after running two aphid homogenates. In *N. ribisnigri* (Nr1A) the BuChE appears in the most cationic portion of the gel (the top).

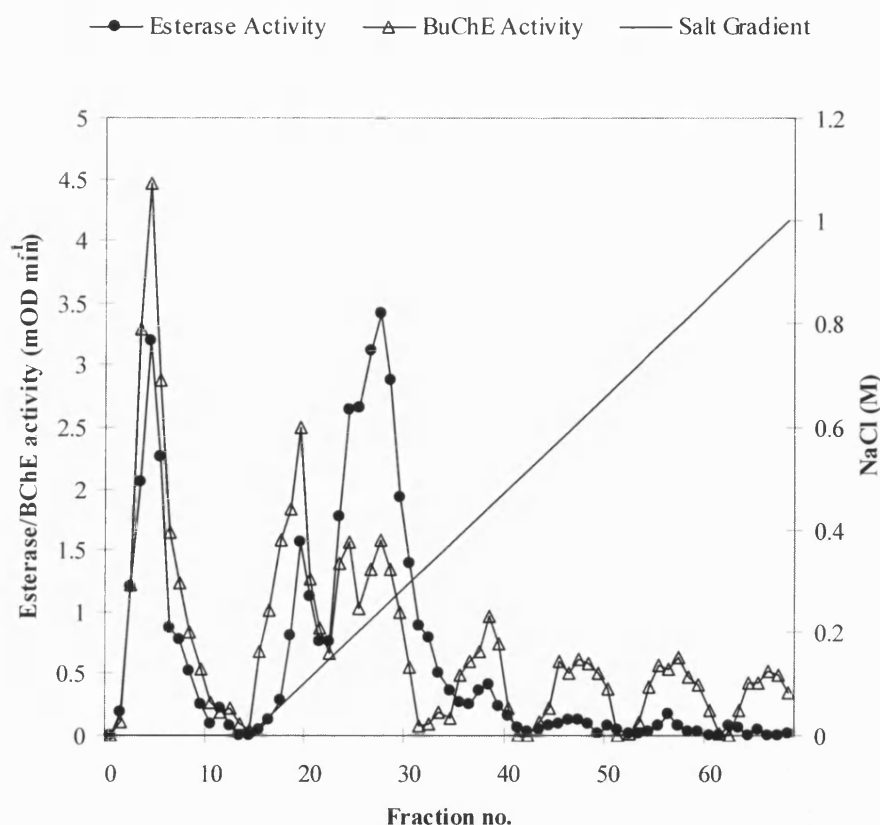


Figure 4.14 Measurement of esterase and BuChE activity in eluted fractions after anion-exchange chromatography.

When single aphids of Nr1A and Nr2A were homogenised and assessed for BuChE activity, the mean activity value of Nr2A was greater than that of Nr1A but as with total esterase values (see Chapter 3), there was an overlap in response by individual S and R aphids (Figure 4.15). The assay also showed around 4-fold increase in BuChE activity at pH9 compared with pH7.

The importance of BuChE in pirimicarb resistance was assessed by measuring BuChE activity in a microplate assay in the presence of pirimicarb (Figure 4.16). While there was a slight inhibition of activity at a very high concentration of pirimicarb (10mM), unlike *N. ribisnigri* AChE or E0.34, BuChE activity was not inhibited by the compound. Although a difference was observed in BuChE activity between Nr1A and Nr2A, because mass homogenates, without any protein measurement, were used in the assay, the significance of the result could not be assessed.

	pH 7		pH9	
	Nr1A	Nr2A	Nr1A	Nr2A
	2.055	5.651	13.802	16.454
	3.943	5.492	12.537	18.192
	3.719	3.138	16.883	18.886
	6.074	2.271	12.173	16.396
	4.410	4.574	10.575	17.931
	2.414	4.037	10.543	10.895
	2.895	4.268	9.500	16.244
	2.264	5.654	12.161	11.027
Mean	3.47	4.39	12.27	15.75
+/- st.dev.	1.36	1.23	2.31	3.11

Figure 4.15 BuChE activity (mOD min^{-1}) in individual *N. ribisnigri* aphids. The standard deviation within populations was too great for the assay to be used as a diagnostic for pirimicarb resistance.

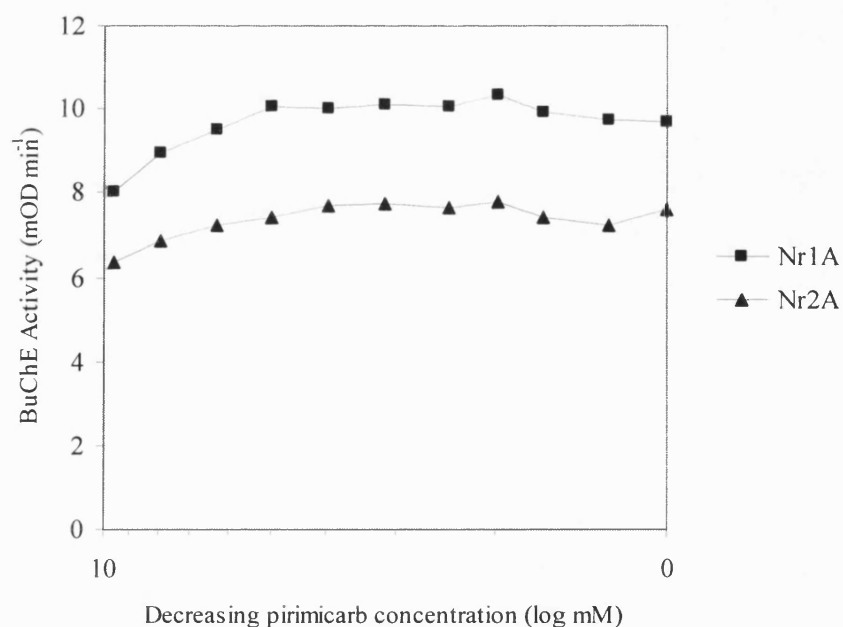


Figure 4.16 Incubation of *N. ribisnigri* homogenates with pirimicarb showed only slight inhibition of BuChE activity at high concentrations (10mM).

4.4. Discussion

The continued correlation between pirimicarb resistance in bioassays and enhancement of the esterase band, E0.34, supported the need to purify this enzyme and conduct further biochemical characterisation in order to understand how the enzyme was conferring resistance.

Initial attempts to purify E0.34 by ion-exchange chromatography were complicated by the identification of two peaks of esterase activity. While this could have been caused by overloading of the column, causing proteins to pass straight through, repeating the experiment with small quantities of crude homogenate and different ion-exchange media showed that both anionic and cationic proteins were present in *N. ribisnigri* which exhibited high esterase activity. In addition, even though E0.34 activity was identified in the anionic peak, the activity was greatly reduced compared to the post G-25 sample that had been kept in the same environmental conditions. The converse would be normally be expected whereby the esterases would be degraded in the post G-25 solution by proteases.

Because E0.34 appeared stable in PAGE gels, electro-elution provided an ideal alternative purification method. Although it was not the most efficient method of purification (using 20g of aphids to produce only 1mg of protein), it allowed the esterase to be purified without requiring any further understanding of the biochemical properties of the enzyme. For the same reason, preparative electrophoresis also proved a reliable method by which to purify E0.34 but with the resolving capabilities of ion-exchange chromatography.

Reactivation work undertaken on crude aphid homogenates was hindered as the role of E0.34 in detoxification could not be distinguished from the activity of the other esterases that the assay detected. Therefore, purification of E0.34 allowed the interaction between the enzyme and pirimicarb to be studied for the first time. The experiment showed the role of the E0.34 in pirimicarb detoxification is mainly one of sequestration rather than significant hydrolysis of the insecticidal ester. The elevation in esterase activity was shown to be due to over-production of the esterase

rather than the presence of a more active form (Chapter 3). Together, the finite reserve of detoxifying esterase coupled with the action of sequestration, probably accounts for the low-level resistance to pirimicarb observed in bioassays (Chapter 2).

The requirement of a detergent (Triton X-100) for optimum E0.34 activity provided another method by which the total esterase screening assay could be improved. The requirement of a hydrophobic environment suggests that E0.34 may be membrane bound.

When a purified sample of E0.34 was run on an ion-exchange column the peak of E0.34 activity was seen to be eluted at 0.6M NaCl as opposed to 0.25M NaCl when using the crude homogenate. Based on the principles of ion-exchange chromatography, this would have been caused by a change in the surface charge of E0.34 between its impure and pure form. While these are only preliminary results, it does suggest that an explanation for difficulties in measuring and purifying E0.34 is that upon homogenisation, E0.34 becomes bound to an inhibiting factor which reduces the enzymes' catalytic activity. The activity is recovered when run on a PAGE gel, which would separate the esterase from the inhibitor. Potentially, therefore, the only method to rapidly screen for actual elevated levels of E0.34 would involve removing the inhibiting factor first.

It is therefore possible that the ELISA did not work because the antibodies were raised to E0.34 in its uninhibited form but when individual aphids are homogenised, much of the esterase would be bound to the inhibiting factor and therefore not be 'recognised' by the antibodies. However, it is more probable that there was insufficient E0.34 to cause a specific immune response in the rabbit.

Both the findings of Chapter three and the result of the ion-exchange purification showed that the crude homogenate contained another protein that exhibited esterase activity. The discovery of a cationic protein that could hydrolyse the same model substrate as an anionic, resistance-associated esterase is a phenomenon that appears not to have been reported before. On further investigation, the cationic protein was identified as BuChE, an enzyme more commonly studied in vertebrates. The role of BuChE is not fully understood but it is thought to act as a scavenger protein, perhaps

protecting AChE (Plattebroze and Broomfield, 2000). In the case of *N. ribisnigri*, however, results suggest it is not involved in resistance because its activity appears unaffected by the presence of pirimicarb. This finding explained why there appeared to be activity in the total esterase assay that was unrelated to the quantity of E0.34 present. This did not, however, explain why the distinction between Nr1A and Nr2A was not clearer in the assay, assuming the 'background' BuChE activity did not contribute more than 50% of the total activity, as suggested by the esterase assay incorporating pirimicarb. However, on IEF gels, BuChE appeared to differ in activity between Nr1A and Nr2A homogenates. A BuChE microplate assay was therefore run on individuals to see if BuChE activity could be used as a diagnostic indicator of pirimicarb resistance. Unfortunately, because both enzymes hydrolyse both substrates, a similar situation to that of the total esterase assay occurred where mean values for Nr2A were greater than Nr1A but individual responses overlapped. Based on our knowledge of the properties of E0.34 and BuChE, the insensitivity of BuChE to pirimicarb and its sensitivity to high Triton X-100 concentrations could be utilised to distinguish between BuChE activity and esterase activity in a microplate assay.

Purification of E0.34 showed:

- The role of E0.34 in pirimicarb resistance was one of sequestration rather than rapid hydrolysis.
- E0.34 required a detergent for optimum activity, suggesting the enzyme is membrane bound.
- Purified E0.34 has a different surface charge from its impure form suggesting something may bind to it upon homogenisation.
- 50% of the total esterase activity in *N. ribisnigri* is attributable to BuChE, an enzyme unaffected by pirimicarb.

5. IMPLICATIONS FOR FIELD CONTROL

5.1. Introduction

Despite a mechanism being identified for pirimicarb resistance in *N. ribisnigri* and clear bioassay evidence of increasing pyrethroid resistance, the findings needed to be related to the control difficulties in the field. There are examples of substantially increased tolerance in bioassays causing little or no reduction in field control efficacy (Farnham *et al.*, 1984) and, conversely, examples of resistance barely detectable in bioassays having a major impact in the field (Dennehy and Granett, 1984). In order for the toxicological and biochemical findings to be applied as part of a resistance management strategy, the impact of the mechanisms in the field needed to be investigated.

Field experiments described below were conducted primarily by staff at Horticultural Research International, Wellesbourne. However, the phenotype of the clones used was characterised by bioassay and PAGE analysis throughout the project at Rothamsted Research.

5.2. Materials and Methods

5.2.1. Field efficacy of pirimicarb and deltamethrin sprays

The first field experiment (conducted in August 2000) tested the effectiveness of pirimicarb and deltamethrin for controlling three clones of *N. ribisnigri* (Nr1A, Nr2A and Nr4A) compared to untreated control plants. Lettuce plants (cv. Saladin) were grown for three weeks in an insect proof cage in a glasshouse until planting into individual plots. Each plot was planted with two rows of ten plants. Plant spacing was 30cm within rows and 45cm between rows. Each plot of 20 plants was covered with an insect proof mesh cage the following day. Each of the nine treatment combinations (three aphid clones x three treatments) were assigned randomly to a single plot in each of six blocks, giving six replicates of each of the nine treatment combinations (54 plots in total).

Once established, each lettuce plant was inoculated with aphids of the appropriate clone by placing a piece of leaf from the bulked-up aphid cultures containing approximately ten aphids in the middle of each plant. Owing to poor weather, inoculated *N. ribisnigri* were allowed to establish for 17 days before the first pre-treatment sample was taken (normally 7 days). The following day plants were treated with either, pirimicarb (0.25g a.i./l at 600l/ha) or deltamethrin (6.25g a.i./l at 600l/ha) or left untreated (control). All treatments were applied by hand held 1.5m boom at a height of 1m above the crop. Plants were then sampled two and six days after treatment. Of the 20 plants in each cage, six were cut, individually bagged and taken to the laboratory on each sampling occasion. The total number of small nymphs per plant was recorded.

Aphid numbers per plot (summed across the six sampled plants per occasion) were analysed using a generalised linear model framework, using a log-linear model assuming Poisson distributed data. Over-dispersion of the counts was allowed for in the analysis. Analyses included the total pre-treatment count (after \log_e transformation) as a covariate to adjust for plot differences in the numbers prior to treatment. Treatment effects estimated in the model are re-expressed in terms of the percentage mortality for each chemical treatment relative to the untreated control.

5.2.2. Effect of pyrethroid resistance on plant infestation levels

The efficacy experiment described in 5.2.1 was repeated in August 2001 using pirimicarb (0.25g a.i./l at 600l/ha) or lambda-cyhalothrin (3.75g a.i./l at 600l/ha) against Nr1A and Nr4A aphids. However, Chi-square analyses were done on the number of plants with 0, <5 or >5 aphids on them 2 and 6 days after treatment in order to compare infestation levels rather than mean total aphid numbers.

5.2.3. Efficacy of pirimicarb and deltamethrin residues

Individual marked leaves of five week old lettuce cv. Saladin were dipped in solutions of pirimicarb ('Pirimor') (0.5g/ L) or deltamethrin ('Decis') (0.42ml/ L) or were left untreated (conducted in August 2000). Degradation of the chemicals outside was allowed for zero, one, two, four and seven days after treatment before being moved to the glasshouse. Each plant was inoculated with ten alate adults of either Nr1A (susceptible), Nr2A (pirimicarb resistant) or Nr4A (pyrethroid resistant)

in two clip cages (five per cage). The nine treatment combinations were replicated five times. Mortality, measured as dead and moribund individuals, was recorded after 48h. Data for percentage mortality were subjected to analysis of variance for each sampling occasion separately. Percentage mortality data were arcsine transformed prior to analysis to stabilise sample variances. Back-transformed means were calculated following each analysis.

5.3. Results

5.3.1. Field efficacy of pirimicarb and pyrethroid sprays

Aphid mortality (restricted here to the most significant data, young nymph numbers) on pirimicarb and deltamethrin treated plants is summarised in Figure 5.1a-b. There was no difference in the number of young nymphs between the susceptible Nr1A clone and the pirimicarb resistant clone, Nr2A, on plants two days after treatment with pirimicarb. However, six days after treatment, mortality of small nymphs for Nr2A was significantly less than for Nr1A and Nr4A ($P < 0.1$). In contrast, there was a significant difference in mortality between clones immediately after deltamethrin treatment with the pyrethroid resistant clone, Nr4A, demonstrating significantly reduced mortality, both two and six days after treatment, compared to the other two clones ($P < 0.1$).

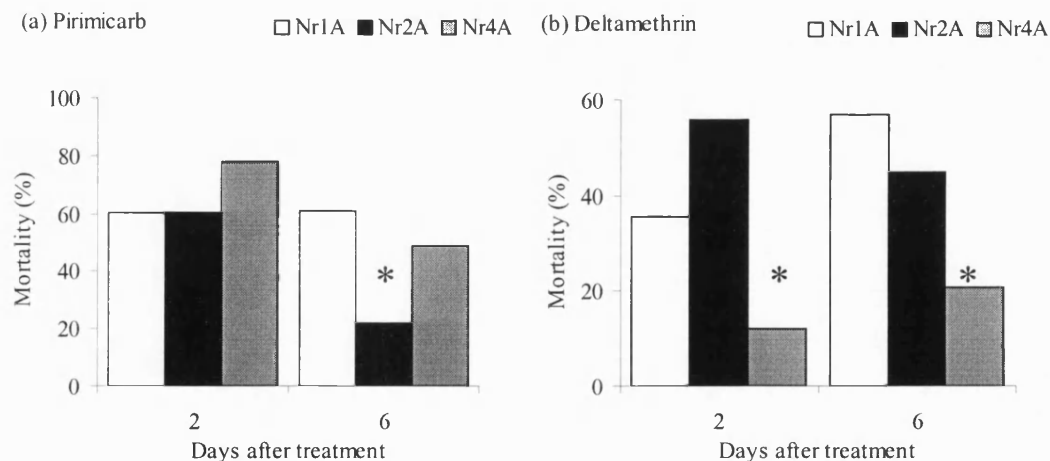


Figure 5.1a-b. Percent mortality of young nymphs two and six days after spraying, related to number on untreated plants. Populations were Nr1A (fully susceptible), Nr2A (pirimicarb resistant) and Nr4A (pyrethroid resistant). Values that were significantly lower ($P < 0.1$) than the respective Nr1A values are indicated by an asterisk.

When the experiment was repeated using Nr1A and Nr4A, the level of control for both clones was very high with no significant difference in mean number of aphids, 2 and 6 days after treatment (data not shown). However, when the number of infested plants was calculated, significant differences (at the 95% level for Chi-square comparisons) between the two clones were seen. Six days after treatment with pirimicarb, Nr4A was found on six plants compared with complete kill in Nr1A (Figure 5.2a). Two days after spraying with lambda-cyhalothrin, Nr4A was found on significantly more plants than Nr1A (10:1 infested plants, Nr4A:Nr1A), which increased to 20:10 (Nr4A:Nr1A) six days after treatment (Figure 5.2b).

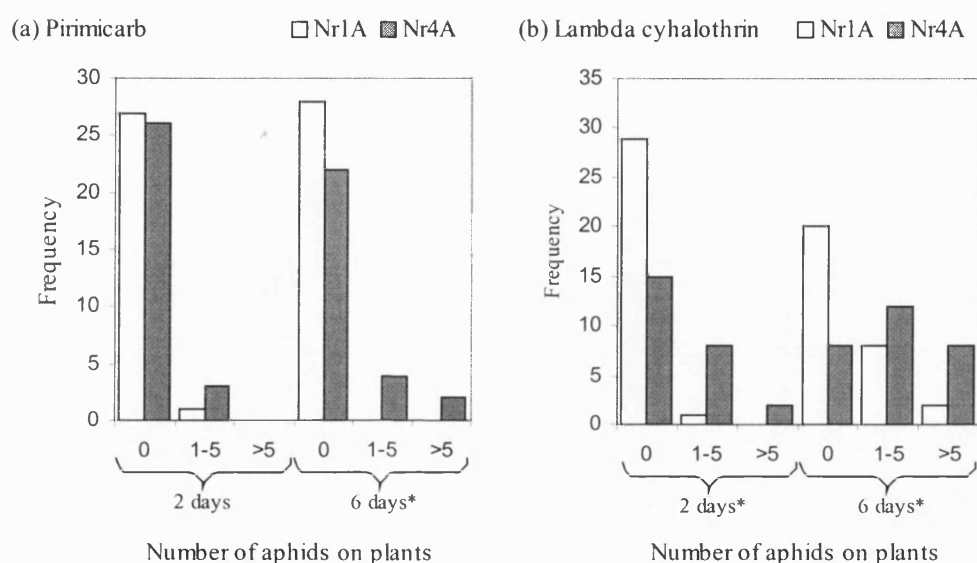


Figure 5.2a-b Number of plants infested with either Nr1A or Nr4A 2 or 6 days after treatment. Times at which there were significant differences between Nr1A and Nr4A values are indicated by an asterisk (at the 95% level based on chi-square comparisons).

5.3.2. Efficacy of pirimicarb and deltamethrin residues

In all three clones, mortality declined with time. Mortality of Nr2A was comparable on both pirimicarb- and deltamethrin-treated plants one day after treatment, being significantly lower than Nr1A. Nr4A exhibited significantly less mortality on deltamethrin-treated plants compared with pirimicarb-treated plants one day after treatment. Two and four days after treatment, mortality was significantly reduced in Nr2A and Nr4A aphids on both treatments compared to Nr1A. Low mortality levels on all treatment combinations were observed seven days after treatment (Figure 5.3a-b).

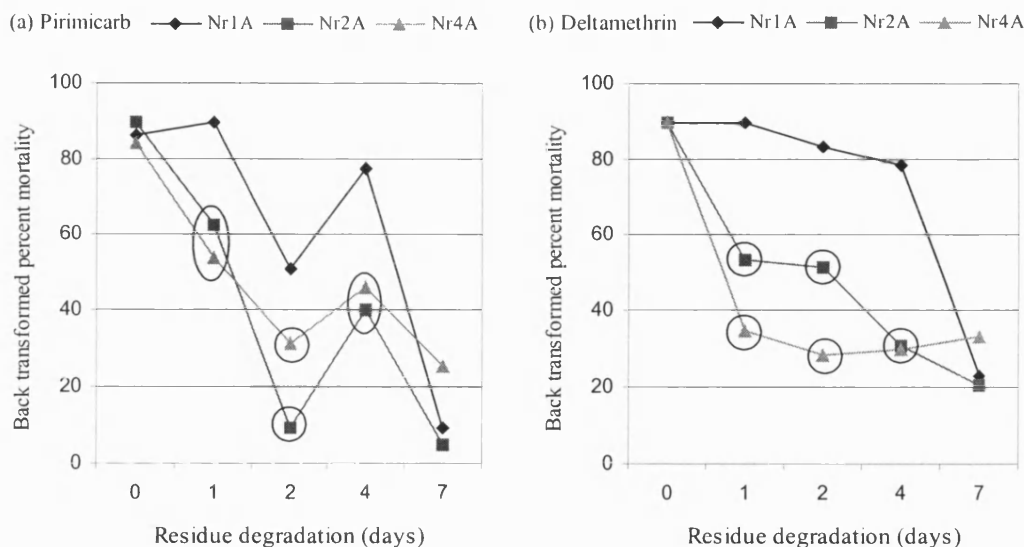


Figure 5.3a-b Back transformed percent mortality of Nr1A, Nr2A and Nr4A 48h after being placed on pirimicarb or deltamethrin residues which had been subjected to different periods of degradation. Circled values indicate significant decreases in mortality compared to Nr1A ($P<0.05$).

5.4. Discussion

Despite the low levels of pirimicarb resistance recorded in laboratory bioassays, the field experiments showed that they did result in reduced control. The results suggest that pirimicarb resistance is expressed primarily in terms of a reduced period over which pirimicarb killed the Nr2A clone effectively. Therefore, when assessing total aphid numbers (nymphs and adults), the difference between Nr1A and Nr2A was not always significant because adults were killed in equal numbers. However, the few Nr2A surviving individuals were able to reproduce earlier leading to a greater nymph population six days after spraying.

In contrast, there was significantly lower initial mortality of Nr4A aphids compared to Nr1A 2 and 6 days after treatment with deltamethrin. However, in repeated experiments, this significant difference was not always apparent. The assessment method was therefore refined to calculate differences in infested plant numbers, providing a result of more direct interest to lettuce growers. This not only confirmed that there was significant control failure both 2 and 6 days after treatment with Nr4A but also showed slightly reduced control six days after pirimicarb treatment.

The residue experiment confirmed the efficacy findings. Nr2A initially showed high mortality until placed on two day degraded residues where survival was high. In contrast, the pyrethroid resistant Nr4A showed high survival after 1 day residue degradation, and this survival rate changed little over time.

To be fully effective against resistant individuals, pirimicarb sprays need to hit the aphid directly. This can be a problem when spraying lettuce plants with a tight head or if the spray applied before a major *N. ribisnigri* attack. Because one aphid on a lettuce is considered to be contamination by the UK lettuce industry, the ratio of infested plants to non-infested plants becomes more important than how many aphids are in the infestation.

Results obtained for *N. ribisnigri* provide some interesting contrasts with the long-studied problem of insecticide resistance in the peach-potato aphid, *Myzus persicae*. All three mechanisms of resistance in the latter confer extremely potent protection against at least one insecticide class, with very obvious consequences for growers. To date, the situation in UK *N. ribisnigri* appears more subtle, with the individual mechanisms being less potent and having a more insidious impact on the efficacy of insecticide treatments. For both pirimicarb and pyrethroids, the most marked effect is on the duration of control achieved rather than a dramatic loss of effectiveness at the time of application. Reasons why such resistance is nonetheless perceived as highly problematical by salad growers undoubtedly include:

- (a) Very low or zero tolerance of aphids on harvested produce, i.e. any survivors at all constitutes an economic risk.
- (b) Difficulties with spray delivery posed by the architecture of lettuce plants, especially late in the growing cycle, which can favour the survival of individuals with only a slight fitness advantage over their susceptible counterparts.
- (c) Re-invasion of lettuce plants after spraying, which was excluded in our field experiments, will accelerate a recovery in aphid numbers and compound difficulties caused by the enhanced survival of aphids present at the time of applications.

In this context, lettuce constitutes a crop at risk from even minor shifts in tolerance that might go unnoticed or be disregarded by growers of other commodities.

The field experiments showed:

- Low level pirimicarb resistance identified by bioassays resulted in significant control failure six days after pirimicarb treatment.
- Pirimicarb resistance was caused by survival on residues rather than spray contact.
- Pyrethroid resistance caused almost immediate control failure.
- Assessment of infested plants rather than total aphid numbers may highlight subtle variations and provide more informative results.

6. GENERAL DISCUSSION AND CONCLUSIONS

6.1. Incidence of Insecticide Resistance in *N. ribisnigri*

Between 1999 and 2001, UK field populations of *N. ribisnigri* exhibited four modes of resistance: (1) full susceptibility; (2) pirimicarb resistance; (3) pyrethroid resistance and (4) pirimicarb and pyrethroid resistance. Comparisons with published data (Barber *et al.*, 1999) showed reduced levels of pirimicarb resistance (from 10-fold to *c.* 3-fold) and increased levels of pyrethroid resistance (from *c.* 10-fold to 25-fold). Resistance to one pyrethroid conferred cross-resistance to the other pyrethroid tested. As the samples were not collected at specific times at specific sites, no conclusions can be made about the development of resistance other than stating that such resistant populations do occur in the UK. The reduction in pirimicarb resistance by the 'standard' pirimicarb clone, Nr2A, probably reflects the time over which the clone had been in culture without selection pressure. Beranek (1974) describes a possible interval timer, unique to different genomes which determines when resistance is 'turned off' in the absence of a selection pressure, given the varied generation times over-which loss of resistance has been reported. Lower pirimicarb resistance in the more recently collected field samples may have been caused by a change in spray regime by growers i.e. reduced pirimicarb use and increased pyrethroid use.

Resistance to imidacloprid is rare in other insect species but has been documented in *B. tabaci* (Cahill *et al.*, 1996; Elbert and Nauen, 2000) and in the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Olson *et al.*, 2000). The potential for insects to be tolerant to imidacloprid is a phenomenon recently reported for *M. persicae* where it was found that tolerant populations are killed by the recommended dose of the compound but were more likely to survive and reproduce at lower concentrations (Foster *et al.*, In Press). The occurrence of similar levels of tolerance in *N. ribisnigri* would have serious implications for the control of this aphid. The use of imidacloprid on UK lettuce is by seed treatment only so while tolerant populations will probably be killed early in the growing season, over time the compound is translocated and diluted around the plant allowing later migrations of tolerant

populations onto the lettuce to survive. This in turn creates a potential to develop more potent resistance in the future.

6.2. Mechanisms of Insecticide Resistance

The occurrence of four different modes of resistance against two types of insecticides provided good evidence that two independent resistance mechanisms were present in *N. ribisnigri*. Pirimicarb resistance was consistently associated with an elevation of E0.34 activity on PAGE gels. The esterase band was found to be lost in resistant aphids subjected to over crowded rearing conditions. At the same time, the aphids became susceptible to pirimicarb in bioassays while retaining pyrethroid resistance (in those clones exhibiting both forms of resistance). This provided further circumstantial evidence for both the role of the E0.34 in pirimicarb resistance and the presence of another mechanism, responsible for pyrethroid resistance.

The cause of this loss of esterase activity could be related to plant secondary metabolites. The choice of plant upon which laboratory cultures are reared has been proven to be important in maintaining resistance levels. For example Yang *et al.* (2001) showed reduced resistance levels to bifenthrin, lambda-cyhalothrin and dimethoate and an associated reduction in esterase activity seven days after moving *T. urticae* from lima bean plants to cucumber. In the case of *N. ribisnigri*, however, because of its monophagous nature, there was no alternative choice of rearing plant. Yu and Abo-Elghar (2000) found GST activity in the fall armyworm, *Spodoptera frugiperda* (Smith) to be inhibited by numerous plant allelochemicals such as flavonoids and other phenols. Phenolic acids such as chlorogenic acid, known to have mild anti-insect effects (Constabel, 1999), are present in lettuce and are induced by wounding (Loiza-Velarde *et al.*, 1997). However, any involvement with esterase inhibition is purely hypothetical and preliminary work running the total esterase assay in the presence of chlorogenic acid has yielded no positive results (data not shown). It is also possible that the reduction of esterase activity and associated loss of resistance is associated with starvation of the aphids in over-crowded conditions and an associated redistribution of biochemical resources.

Pirimicarb resistance was conferred by overproduction of E0.34 and sequestration of the compound by the esterase. Although characterisation of pirimicarb S and R individuals by total esterase assessment was improved by extending the assay run time and by the addition of Triton X-100 to the reaction, the assay did not provide sufficient distinction between populations to be used as a rapid screening assay. However, the frequency of responses by the S and R clones do show normal distribution curves within the populations. When compared with the responses of *M. persicae* reported by Sawicki *et al.* (1980), the overlap in total esterase activity between Nr1A and Nr2A is similar to that of the *M. persicae* clones US1L and MS1G. Given the low pirimicarb resistance levels of Nr2A, this result is therefore not surprising and the diagnostic assay may prove more useful in the future if populations of greater pirimicarb resistance arise. However, it was the discrepancy between the clear distinction between Nr1A and Nr2A, based on E0.34 activity, on PAGE gels and the poor distinction by the total esterase assay that led to further characterisation of the enzyme.

Concerns about which enzymes were being determined by total esterase assays were raised as far back as 1953 by Aldridge. At this time, esterases identified in blood sera were categorised into A-esterases, which were not inhibited by the substrate *p*-nitrophenyl phosphate (E 600), and B-esterases, which were very sensitive to inhibition by 10^{-8} M E 600. Greater hydrolysis of *p*-nitrophenyl acetate (similar to 1-naphthyl acetate used in this study) was observed in A-types compared to B-types and *vice-versa* for activity against *p*-nitrophenyl butyrate.

In *N. ribisnigri*, inhibition studies with pirimicarb provided the first indication that E0.34 contributed only 50% of the total esterase activity, a situation described by van Asperen (1962) in *M. domestica* (see 1.2.1.1.1). The use of pirimicarb in the present experiment precluded the involvement of AChE in the assay. However, Brestkin *et al.* (1985) identified two cholinesterases in the spring grain aphid, *Schizaphis graminum* (Rondani). One, BuChE, was found only in the soluble fraction of the aphid homogenate while Triton X-100 was required to solubilise the AChE from the cell structures. This finding correlated to that of *N. ribisnigri* whereby optimal BuChE activity was identified on IEF gels that had not been pre- or post-treated with Triton X-100. As the experiments of Brestkin *et al.* (1985) concerned the separation of

AChE from BuChE, this solubility difference was used for purification purposes rather than ion-exchange chromatography. In addition, they utilised the fact that unlike vertebrate BuChE and other cholinesterases, aphid BuChE does not hydrolyse ATChI and so specific inhibitors are not required. They do not, however, make reference to the possible involvement of general esterases with either ATChI or BTChI.

In the current project, the distinction needed to be made between BuChE and general esterases, rather than AChE, as although BuChE contributed *c.* 50% of total esterase activity, it didn't appear to have a role in resistance. Unlike aphid AChE, there is no known inhibitor for insect BuChE. Although some are known for human BuChE, such as tetraisopropyl pyrophosphoramidate (iso-OMPA) (Rakonczay *et al.*, 2001), preliminary experiments (data not shown) suggested that this compound does not affect aphid BuChE. The strong cationic surface charge of BuChE in *N. ribisnigri* did allow the enzyme to be clearly separated from other esterases (all with anionic charges) by both ion-exchange purification and IEF.

It has been previously suggested that insects may only have one AChE with mixed properties of vertebrate AChE and BuChE (Gnagey *et al.*, 1987; Toutant, 1989; Yerushalmi and Cohen, 2002). This project provides circumstantial evidence for a categorical distinction between the two cholinesterases. However, because the focus of the project was on differentiating BuChE from esterases, further work would have to be conducted focusing on the distinction between AChE and BuChE to prove this conclusively. The conclusions of the work with BuChE are that its activity in the total esterase assay contributed to the poor characterisation of S and R individuals by that method, compared to PAGE analysis.

Another factor relevant to the rapid screening of E0.34 activity was the reversible inhibition of the esterase upon homogenisation. Although this theory is still circumstantial, based on the different surface charges of E0.34 in a pure and impure form, it does suggest that E0.34 was relatively inactive in the assay compared to its uninhibited activity, as seen on PAGE gels.

In addition, although the importance of a detergent has been thoroughly described in relation to AChE activity (see above), its use for optimal esterase activity appears to be overlooked in the literature. This issue was raised by Valles *et al.* (2000) regarding a possible microsomal esterase associated with cypermethrin resistance in *B. germanica*. Further work demonstrated the importance of Triton X-100 in solubilising the esterase which was potentially involved in the resistance (Valles and Strong, 2001). The present project is in agreement with their conclusions that ‘the contribution of membrane-bound esterases in insecticide resistance is often overlooked or ignored’.

Despite the difficulties involved with purifying and characterising E0.34, the process highlighted three unusual biochemical features, which have implications for the transfer of biochemical techniques between species. Firstly, if the total esterase assay had been used for esterase analysis in place of the slower PAGE analysis, it is probable that the role of E0.34 in pirimicarb resistance would have been overlooked. Secondly, normal PAGE analysis favours the identification of anionic proteins and standard IEF analysis protocols recommend placing application pieces only 1cm away from the cathode thus reducing the chance of clearly visualising cationic bands. Finally, if the pure E0.34 sample had not been passed through the ion-exchange column to determine its elution profile, the altered surface charge would not have been found. Without deviating from traditional methods and protocols, the problems associated with characterising pirimicarb resistance in *N. ribisnigri* would not have been identified.

These findings can tentatively be applied to the work of Rufingier *et al.* (1999) who correlated higher non-oxidative and oxidative degradations with pirimicarb-resistant population on the basis of synergism studies with piperonyl butoxide (PBO). Resistance levels remaining after synergism were related to target-site insensitivity. No significant difference was found in the ability of S and R in the total esterase assay. Based on the findings of the current project, that elevated esterases are not easily identified in *N. ribisnigri* by rapid assay, and that PBO can inhibit esterases (Gunning *et al.*, 1998), it is more probable that resistance to pirimicarb in the French and Spanish populations was due to insensitive AChE, coupled with elevated esterases.

Owing to time limitations, detailed studies of all resistance related enzymes in this project could not be conducted . Therefore, the findings have implications for the conclusions drawn from other aspects of this project which were examined in less detail such as the GST assays, which were conducted using standard protocols without detailed studies of the enzymes involved. Similarly, only one region of the sodium channel was examined to implicate *kdr* as the mechanism of pyrethroid resistance on the basis of work with *M. persicae* and other insect pests. It is possible that mutations could exist in any part of the *N. ribisnigri* sodium channel, which confer pyrethroid resistance without a fitness cost. For example, two mutations were found in the domain I to II linker gene of *B. germanica* which, in association with the ‘normal’ *kdr* mutation in domain II of the sodium channel, conferred high pyrethroid resistance (Tan *et al.*, 2001).

6.3. Control of Resistant *N. ribisnigri*

Despite the low-levels of pirimicarb resistance reported in bioassays, control problems were demonstrated six days after spraying when resistant individuals that avoided direct spray contact were able to survive on pirimicarb residues and reproduce more successfully than the susceptible population. In contrast, pyrethroid resistance conferred higher survival under both direct spray and residue contact, however, neither mechanism resulted in dramatic loss of control at the time of application. On the basis of these findings, the advice released to growers included standard resistance prevention procedures such as following dose recommendations and rotation of insecticide compounds. In addition, more specific information suggested alternating between pirimicarb and a pyrethroid to counter single resistant individuals, optimising pirimicarb sprays to ensure maximum kill at the time of spraying as well as strategic use of the pirimicarb/lambda-cyhalothrin mixture, ‘Dovetail’. As imidacloprid is applied as a seed treatment, no change in use was recommended but owing to a potential risk of tolerance developing to this compound, growers were advised to report control failure immediately. The risk of resistance to this compound will be increased if the new neonicotinoid, thiacloprid, is granted a SOLA (HDC News, 2002). However, new compounds are urgently required for the protection of lettuce and the availability of such a compound would

be well received by growers. One such compound is pymetrozine, recently approved for SOLA use on lettuce. Although preliminary bioassay results suggest this compound is not completely effective against adult *N. ribisnigri*, owing to the anti-feedant nature of the compound, field efficacy could be improved by targeting developing nymphs early in the growing season before infestations become established.

6.4. Future Studies

The current project was designed to confirm the resistance status of *N. ribisnigri* populations in the UK and provide advice to lettuce growers on how to manage the problem. An assessment of the spatial and temporal dynamics of resistance levels was therefore not possible based on the sporadic collection of samples. Any future monitoring experiments should therefore involve systematic collection of samples at specified times and locations in order to collate such data. Connected with this work is the possible decline of pirimicarb resistance in culture in the absence of a selection pressure. Again, a more thorough and systematic approach, similar to that of Sawicki *et al.* (1980) when investigating the stability of esterase-based resistance in *M. persicae*, is needed in order to monitor this phenomenon in relation to generation time.

Insecticide resistance is sometimes associated with fitness costs (e.g. Foster *et al.*, 2000). Although no specific fitness cost study was conducted during this project, preliminary observations from the field trials showed significantly higher numbers of the pyrethroid-resistant clone when compared with the susceptible clone on untreated plants. As these results were obtained with single clones it may be that the greater capacity for increase is unrelated to insecticide resistance. A direct relationship with a possible fitness advantage could only be determined by further investigations into the capacity for increase of more than one clone for each insecticide resistance characteristic, so that a range of data can be obtained.

In terms of resistance biochemistry, the discovery of BuChE in *N. ribisnigri* has opened many new channels of investigation. Although little work has been conducted on insect BuChE, the implications of its unique properties are far

reaching. There is much interest in the use of the enzymes as bioscavengers to protect humans from OPs used in chemical warfare (Plattebroze and Broomfield, 2000). The two problems associated with this technique are producing enough BuChE to be of commercial use and successful purification of the enzyme. The advantage of the *N. ribisnigri* BuChE is the ease of purification because it appears very stable and is the only cationic protein that exhibits BuChE/esterase activity within the crude aphid homogenate. Problems of investigating AChE and BuChE were raised by Brestkin *et al.* (1985) who observes that because dissection of an aphid in order to retrieve AChE rich tissue from an aphid is not an option, a good purification method is required. Gao and Zhu (2001) state that ‘to date, neither AChE nor BuChE has been purified to a substantial degree from any aphid species’ This was partially resolved by the authors, who purified and sequenced AChE from *S. graminum*. The simple purification of *N. ribisnigri* BuChE provides an opportunity to complete such studies.

The present project was designed to characterise insecticide resistance in UK populations in *N. ribisnigri*. The key comparison species was *M. persicae* for which thirty years of resistance research exists. However, while bioassay techniques and potential control strategies differ little between the two species, the low levels of pirimicarb resistance and the presence of an esterase-inhibiting factor in *N. ribisnigri* precluded a simple transfer of techniques between species. The results of this project have not only allowed resistance management guidelines to be produced as well as providing supporting data for SOLA applications, they have presented serious questions regarding the interpretation of biochemical resistance data in different insect species.

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